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(54) Title: METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF HEMATOPOIETIC GROWTH FACTOR GENES

(57) Abstract

The invention provides a method of effecting expression of growth factors in cells or in multicellular animals and methods for testing compounds as effectors of transcription of growth factors.

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METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF HEMATOPOIETIC GROWTH FACTOR GENES

This application is a continuation-in-part of U.S. Serial No. 644,246, filed January 18, 1991, the contents of which are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Hematopoiesis

The cells of the circulatory system (erythrocytes, various white blood cells and the cells which produce platelets) are all developmentally derived from a common precursor cell type, the pluripotent bone marrow stem cell (1).

The developmental pathway taken by any single stem cell is directed by the activities of a family of circulating glycoprotein hormones known collectively as hematopoietic growth factors (the process of blood cell development is called hematopoiesis) (2). The family of known hematopoietic growth factors includes erythropoietin (EPO), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Granulocyte Colony Stimulating Factor (G-CSF), Macrophage

Colony Stimulating Factor (M-CSF), and the various Interleukins, particularly Interleukin 3 (IL-3) and Interleukin 7 (IL-7) (3). The levels of these growth factors are thought to respond to the organism's needs and bring about required changes in the cellular makeup of the blood. For example, hypoxia induces expression of the erythropoietin gene, which in turn directs the development of more erythroid progenitor cells into erythrocytes, increasing the oxygen carrying capacity of the blood thereby relieving the hypoxia (4).

The activities of many of the hematopoietic growth factors are enhanced synergistically by a recently characterized protein hormone called stem cell factor (SCF) (5). SCF is the product of the Steel (Sl) locus first identified as a developmental allele in mice (6). SCF is produced by the stroma of the bone marrow and by various other cell types (7). It acts directly on the stem cell population by binding to, and activating a membrane spanning receptor coded for by the murine white spotting locus (W), recently found to code for the c-kit proto-oncogene (8). The SCF ligand presumably activates (potentiates) stem cells by altering c-kit's protein tyrosine kinase activity, a mechanistic feature shared by other (perhaps most) hematopoietic growth factor receptors (9).

Pharmaceuticals with which the physician can manipulate the hematopoietic system would be very useful for treating anemia, neutropenia and fighting opportunistic infections. The hematopoietic growth factors themselves have shown great potential as protein therapeutic agents. The estimated market for recombinant hematopoietic growth factors exceeds 2 billion dollars a year.

In spite of their great potential, protein based

pharmaceuticals suffer from several general limitations; they need to be delivered by injection, they are unstable (affecting shelf-life) and they are very expensive to manufacture. Here we describe a method to find small molecular weight organic compounds, which when administered in vivo have the same biological consequences as the hematopoietic growth factors. The general approach is to screen compound libraries for substances which increase expression of the endogenous hematopoietic growth factor genes.

The expression of a specific gene can be regulated at any step in the process of producing an active protein. Modulation of total protein activity may occur via transcriptional, transcript-processing, translational or post-translational mechanisms. Transcription may be modulated by altering the rate of transcriptional initiation or the progression of RNA polymerase (11). Transcript-processing may be influenced by circumstances such as the pattern of RNA splicing, the rate of mRNA transport to the cytoplasm or mRNA stability. This invention concerns the use of molecules which act by modulating the in vivo concentration of their target proteins via regulating gene transcription. The functional properties of these chemicals are distinct from previously described molecules which also affect gene transcription.

Researchers have documented the regulation of transcription in bacteria by low molecular weight chemicals (12,13). Extracellular xenobiotics, amino acids and sugars have been reported to interact directly with an intracellular proteinaceous transcriptional activator or repressor to affect the transcription of specific genes (14).

Transcriptional regulation is sufficiently different

between procaryotic and eucaryotic organisms so that a direct comparison cannot readily be made. For example, procaryotic cells lack a distinct membrane bound nuclear compartment. Furthermore, the structure and organization of procaryotic DNA elements responsible for initiation of transcription differ markedly from those of eucaryotic cells.

The eucaryotic transcriptional unit is much more complex than its procaryotic counterpart and consists of additional elements which are not commonly found in bacteria, including enhancers and other cis-acting DNA sequences (15,16). Procaryotic transcription factors most commonly exhibit a "helix-turn-helix" motif in the DNA binding domain of the protein (17,18). Eucaryotic transcriptional factors frequently contain a "zinc finger" (18,19) a "helix-loop-helix" or a "leucine zipper" (20) in addition to sometimes possessing the "helix-turn-helix" motif (21). Furthermore, several critical mechanisms at the post-transcriptional level such as RNA splicing and polyadenylation are not found in procaryotic systems (22,23).

In higher eucaryotes, modulation of gene transcription in response to extracellular factors can be regulated in both a temporal and tissue specific manner (24). For example, extracellular factors can exert their effects by directly or indirectly activating or inhibiting tissue specific transcription factors (24,11).

Modulators of transcription factors involved in direct regulation of gene expression have been described, and include those extracellular chemicals entering the cell passively and binding with high affinity to their receptor-transcription factors. This class of direct

transcriptional modulators include steroid hormones and their analogs, thyroid hormones, retinoic acid, vitamin D_3 and its derivatives, and dioxins, a chemical family of polycyclic aromatic hydrocarbons (19,23,26).

Dioxins are molecules generally known to modulate transcription, however, dioxins bind to naturally-occurring receptors which respond normally to xenobiotic agents via transcriptionally activating the expression of cytochrome P450, part of an enzyme involved in detoxification. Similarly, plants also have naturally occurring receptors to xenobiotics to induce defense pathways. For example, the fungal pathogen Phytophthora megasperma induces an anti-fungal compound in soybeans. Such molecules which bind to the defined ligand binding domains of such naturally occurring receptors are not included on the scope of this invention.

The clinical use of steroid hormones, thyroid hormones, vitamin D_3 and their analogs demonstrates that agents which modulate gene transcription can be used for beneficial effects, although these agents can exhibit significant adverse side effects. Obviously, analogs of these agents could have similar clinical utility as their naturally occurring counterparts by binding to the same ligand binding domain of such receptors. These types of molecules do not fall within the scope of this invention because they function by binding to the ligand-binding domain of a receptor normally associated with a defined physiological effect.

Indirect transcriptional regulation involves one or more signal transduction mechanisms. This type of regulation typically involves interaction with a trans-membrane signal transducing protein, the protein being part of a multistep

intracellular signaling pathway, the pathway ultimately modulating the activity of nuclear transcription factors. This class of indirect transcriptional modulators include polypeptide growth factors such as platelet-derived growth factor, epidermal growth factor, cyclic nucleotide analogs, and mitogenic tumor promoters such as PMA (27,28,29).

It is well documented that a large number of chemicals, both organic and inorganic, e.g. metal ions, can non-specifically modulate transcription. Most heavy metals modulate gene expression through receptors in a mechanism similar to that employed by dioxin, steroid hormones, vitamin D3 and retinoic acid.

Researchers have used nucleotide analogs in methods to non-specifically modulate transcription. The mechanism involves incorporating nucleotide analogs into nascent mRNA or non-specifically blocking mRNA synthesis. Similarly, researchers have used alkylating agents, e.g. cyclophosphamide, or intercalating agents, e.g. doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl CoA reductase, e.g. lovastatin, are known to indirectly modulate transcription by increasing expression of hepatic low density lipoprotein receptors as a consequence of lowered cholesterol levels.

Signal effector type molecules such as cyclic AMP, diacylglycerol, and their analogs are known to non-specifically regulate transcription by acting as part of a multistep protein kinase cascade reaction. These signal effector type molecules bind to domains on proteins which are thus subject to normal physiological regulation by low molecular weight ligands (30,31).

The specific use of sterol regulatory elements from the LDL receptor gene to control expression of a reporter gene has recently been documented in PCT/US88/10095. One aspect of PCT/US88/10095 deals with the use of specific sterol regulatory elements coupled to a reporter as a means to screen for drugs capable of stimulating cells to synthesize the LDL receptor. PCT/US88/10095 describes neither the concept of simultaneously screening large numbers of chemicals against multiple target genes nor the existence of transcriptional modulators which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the hematopoietic growth factor genes, and (c) bind to a protein through a domain of such protein which is not a defined ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand- binding domain is normally associated with a defined physiological or pathological effect. The main focus of PCT/US88/10095 is the use of the sterol regulatory elements from the LDL receptor as a means to inhibit expression of toxic recombinant biologicals.

A study of the interaction of various known transcription factors with the promoter regions of the hematopoietic growth factor genes, and a comparison of their promoter sequences indicates that a subset (GM-CSF, G-CSF, IL-3 and IL-5) of these growth factor genes are regulated, in part, by the activity of a transcription factor (NF-GMa) binding to a common motif, the cytokine-1 site (CK-1; GRGR/TTTY/ACY/AN), although other, as yet undescribed, transcription factors must act on different subsets of the hematopoietic growth factor gene to generate each gene's unique patten of expression (33). Furthermore, in addition to regulation of transcription initiation, it is clear that mRNA stability of many hematopoietic growth factor genes can be specifically regulated (34).

The use of molecules to specifically modulate transcription of a hematopoietic growth factor gene as described herein has not previously been reported and its use will bring surprise since available literature does not propose the use of a molecule, as described, in a method to specifically modulate transcription. Instead, the available literature has reported methods which define domains of transcriptional regulating elements of a hematopoietic growth factor genes.

Further, the practice of using a reporter gene to analyze nucleotide sequences which regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define domains of transcriptional regulatory elements of a gene-of-interest. Reporter genes which express proteins, e.g. luciferase, are widely utilized in such studies. Luciferases expressed by the North American firefly, Photinus pyralis and the bacterium, Vibrio fischeri were first described as transcriptional reporters in 1985 (34,35). Reporter genes have not been previously used to identify compounds which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene encoding the hematopoietic growth factor, and (c) bind to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

A method to define domains of transcriptional regulating elements of a gene-of-interest typically has also involved use of phorbol esters, cyclic nucleotide analogs, concanavalin A, or steroids, molecules which are commonly known as transcriptional modulators. However, available

literature shows that researchers have not considered using a transcription screen to identify specific transcriptional modulators. Apparently, success would be unlikely in doing so, however, we demonstrate herein that this is not the case.

There is utility in developing the method of transcriptional modulation of hematopoietic growth factor genes by using such molecule as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors.

Problems associated with the therapeutic use of recombinant biological factors include the technical difficulties of large scale protein purification, the high costs of protein production, the limited shelf-life of most proteins and in some cases a short biological half-life of the administered protein in the organism. Additionally, therapeutic delivery of proteins normally requires injection. The method described herein provides a means of up-regulating the expression of proteins which are not readily amenable to administration as injectable biologicals.

Furthermore, molecules specifically regulating the activity of one member of a group of closely related proteins are difficult to identify. Bioactive molecules, structurally related at the protein level, may possess distinct regulatory elements at the DNA level which control their expression. Thus, molecules such as the chemical transcriptional modulators defined herein can provide a greater opportunity for specifically modulating the activity of structurally related proteins.

Finally, the molecules described herein may also serve to

mimic normal physiological response mechanisms, typically involving the coordinated expression of one or more groups of functionally related genes. Therefore, determining whether a molecule can specifically transcriptionally modulate the expression of a hematopoietic growth factor gene and the ultimate clinical use of the molecule provides a therapeutic advantage over the use of single recombinant biologicals, or drugs which bind directly to the final target protein encoded by the gene-of-interest.

Summary of the Invention

The invention provides a method for directly transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the hematopoietic growth factor encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene encoding the hematopoietic growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

This invention further provides for a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA which consists essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the hematopoietic growth factor, and (iii) a DNA sequence encoding a polypeptide other than the hematopoietic growth factor, which polypeptide is

capable of producing a detectable signal. The DNA sequence is coupled to, and under the control of, the promoter, and the contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable detectable signal to be produced by the polypeptide so expressed. This allows for a quantitive determination of the amount of the signal produced. By comparing the amount of detectable signal produced with the amount of produced signal detected in the absense of any molecule being tested or upon contacting the sample with any other molecule, this method allows one to identify the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

The invention still further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the gene encoding the hematopoietic growth factor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable change in the amount of the

polypeptide produced, and quantitatively determining the amount of the polypeptide produced. By comparing the amount so determined with the amount of polypeptide produced in the absense of any molecule being tested or upon contacting the sample with any other molecule, the molecule is identified as one which causes a change in the amount of polypeptide expressed, and thus identified as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

The invention further encompasses a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each of the cells so contacted comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of gene encoding the hematopoietic growth factor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter. The contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. The amount of the mRNA produced is quantitatively determined and the amount so determined compared with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to identify the molecule as one which causes a change in the detectable mRNA amount of, and thus

identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

A screening method is also provided. This screening method comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested.

Also disclosed is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding hematopoietic growth factors which comprises essentially simultaneously screening the molecules against the hematopoietic growth factors according to the methods mentioned above.

A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding an hematopoietic growth factor, the expression of which is associated with a defined physiological or pathological effect in the organism, is also included. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene encoding the hematopoietic growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally

associated with a defined physiological or pathological effect.

Brief Description of the Figures

Figure 1 is a view of the mammalian expression shuttle vector pUV102 with its features. The mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions and the insertion of a neomycin resistance gene coupled to the herpes simplex virus thymidine kinase promoter (TK-NEO).

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pD0432 which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, Photinus pyralis and the mouse mammary tumor virus long terminal repeat.

Figure 5 provides the nucleotide sequences of six oligonucleotides, pUV-1 through pUV-6, which were annealed, ligated, and inserted into the SalI/EcoR1 sites of the plasmid pTZ18R.

Figure 6 is a diagrammatic representation of the construction of the plasmid pUV001 from the plasmids pTZ18R and pBluescript KS(+).

Figure 7 is a diagrammatic representation of the construction of the plasmid pUV100 from the plasmid pUV001 and two DNA fragments, the XbaI/XmaI fragment from pMLuci and the XmaI/BamHI fragment from pMSG.

Figure 8 is a diagrammatic representation of the construction of the plasmid pUV100-3 from the plasmid pUV100 and a 476 b fragment containing a dimeric SV40 polyadenylation site.

Figure 9 is a diagrammatic representation of the construction of the plasmids pUV102 and pUV103 from the plasmid pUV100-3 and D-link oligonucleotides and the plasmid pUV100-3 and R-link oligonucleotides, respectively.

Figure 10 provides the nucleotide sequences of oligos 1-4 used for the construction of a synthetic HSV-thymidine kinase promoter and provides a diagrammatic representation of the HSV-TK promoter.

Figure 11 is a diagrammatic representation of the construction of the plasmid pTKL100 which contains the luciferase gene from the firefly, Photinus pyralis and the HSV-TK promoter sequence.

Figure 12 is a diagrammatic representation of the construction of the plasmid pTKNEO which contains the neo gene, from about 3.5 kb NheI/XmaI fragment from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region from pRSVNEO.

Figure 13 is a diagrammatic representation of the construction of the plasmid pTKNEO2 from the plasmid pTKNEO and the oligonucleotides Neo 1 and 2.

Figure 14 is a diagrammatic representation of the construction of the plasmid pTKNEO3 from the plasmid PTKNEO2 and about 0.9 kb EcoRl/SalI fragment from pMClNEO.

Figure 15 is a partial restriction enzyme cleavage map of the plasmid pJM710 which contains G-CSF upstream sequences.

Figure 16 is a partial restriction enzyme cleavage map of the plasmid pGEM5-Luci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 17 is a partial restriction enzyme cleavage map of the plasmid PG-Luc 1 which contains both the luciferase gene from the firefly, Photinus pyralis, and G-CSF upstream sequences.

Figure 18 is a diagrammatic representation of the construction of pGUC84 from oligonucleotides containing the G-CSF leader sequence from +15 to the ATG cloned into the plasmid pUC19.

Figure 19 is a diagrammatic representation of the construction of pGUV100 from the NcoI/ScaI fragment from pGUC84 containing G-CSF leader sequences cloned into the plasmid pUV100.

Figure 20 is a diagrammatic representation of the construction of pGUV1 from the Pst I fragment of the G-CSF promoter from the plasmid pJM710 inserted into the Pst I site in pGUV 100.

Figure 21 is a diagrammatic representation of the construction of the plasmid pGUV-2 by insertion of more G-CSF upstream sequences from pJM710 into pGUV1.

Figure 22 is a diagrammatic representation of the construction of the plasmid pGUV140 from the XbaI fragment from pGUV2 containing the G-CSF-luciferase fusion and the plasmid pUV103.

Figure 23 is a diagrammatic representation of the construction of the plasmid pGUV150 from the ScaI/XbaI fragment from pGUV140 which contains the G-CSF-luciferase fusion and the plasmid pTKNEO-103.

Figure 24 is a partial restriction enzyme cleavage map of a human genomic clone which contains the entire GM-CSF coding region.

Figure 25 is a diagrammatic representation of the construction of the plasmids pGMLS102 and pGMLS103 from plasmid pUV 102 and a 0.7 kb fragment from pGM-2 and from pUV 103 and a 0.7 kb fragment from pGM-2, respectively.

Figure 26 provides the nucleotide sequence of oligonucleotides 1 through 4 and provides a diagrammatic representation of GM-CSF upstream sequences fused with the ATG of the coding region of the luciferase gene from the firefly, Photinus pyralis.

Figure 27 is a diagrammatic representation of the construction of plasmids pGMLL102 and pGMLL103 from the plasmid pGMLS102 and the GM-CSF clone and the plasmid pGMLS103 and the GM-CSF clone, respectively.

Figure 28 is a diagrammatic representation of the construction of the plasmid CSF1-pTZ18 from the plasmid pTZ18R and a gene fragment comprising the first exon and 5' flanking region of M-CSF.

Figure 29 is a diagrammatic representation of the construction of the plasmid p100-RH from the plasmid pUV100 and the gene fragment comprising the first exon and 5' flanking region of M-CSF from the plasmid CSF1-pTZ18.

Figure 30 is a diagrammatic representation of the construction of the plasmid p100-2 by insertion of a PSTI/PVUII fragment from CSFI-pTZ18 and synthetic oligonucleotides into the plasmid pUV100.

Figure 31 is a diagrammatic representation of the construction of p100-RHC by insertion of short M-CSF 5' upstream sequences fused to the luciferase coding sequence into the plasmid p100-RH containing a longer M-CSF 5' upstream fragment.

Figure 32 is a diagrammatic representation of the construction of plasmid pCSF1-102, the M-CSF reporter vector.

Figure 33 is a diagrammatic representation of the construction of the plasmid pEP-7.5B from a 7.5 kb BamH1 fragment consisting of 6.2 kb of the EPO promoter region and the first three EPO exons and the plasmid Bluescript KS(+).

Figure 34 is a diagrammatic representation of the construction of the plasmid pRE from oligonucleotides EPO 9-12 and the plasmid pUV100.

Figure 35 is a diagrammatic representation of the construction of the plasmid pUV2-EP6 from a 6 kb fragment of EPO upstream sequences and the plasmid pUV 102.

Figure 36 is a representation of the plasmid pEPORF106, an EPO reporter vector wherein the ORF1 has been inactivated by oligonucleotide mutagenesis.

Figure 37 is a representation of pEPOEX106, and EPO reporter vector fusing the luciferase coding sequence to

the second exon of the epo gene. ORF1 is inactive due to a mutation in the initiation codon.

Figure 38 is a diagrammatic representation of the construction of the plasmid IL-3-pTZ18R from IL-3 upstream sequences and the plasmid pTZ18R.

Figure 39 is a diagrammatic representation of the construction of the plasmid pTZ-IL-3-Luci from oligonucleotides IL-lu-1 to IL-lu-4, pUV-3 and pUV-6 and the plasmid IL3/PstI-pTZ18R.

Figure 40 is a diagrammatic representation of the construction of the plasmid pTZ-IL3-C from a 500 kb NcoI/EcoR1 fragment from pTZ-IL-3-Luci and the plasmid IL-3-pTZ18R.

Figure 41 is a diagrammatic representation of the construction of the plasmid pUV-IL-3 from a 6.4 kb Hind III/XbaI fragment from pTZ-IL3-C and the plasmid pUV102.

Figure 42 is partial restriction map of plasmid pSCF106 wherein the upstream regulator elements for the stem cell factor genes are fused to the luciferase coding region.

Figure 43 is a partial restriction map of plasmid pUXLuci, a vector used in the contraction of the human growth hormone reporter vector.

Figure 44 is a partial restriction enzyme cleavage map of the plasmid phGH:CAT which contains the CAT gene and human growth hormone promoter sequences.

Figure 45 is a partial restriction enzyme cleavage map of the plasmid phGH-Luci which contains the luciferase gene

from the firefly, Photinus pyralis and human growth hormone promoter sequences.

Figure 46 is an autoradiogram of PCR reactions detecting varying amounts of M-CSF mRNA and a constant amount of lambda DNA.

Figure 47 is an interpretation of the data presented in Figure 46. Relative band intensity of the M-CSF band is plotted against the initial M-CSF concentration.

Figure 48 is an autoradiograph of a Southern blot illustrating the correct integration of luciferase fusion constructs containing the G-CSF, M-CSF and GM-CSF promoters, respectively, into the genomes of 5637 cells (G- and GM-CSF) and HL 60 cells (M-CSF). Lanes designated 5637 and HL 60 had been loaded with DNA preparations from the parental cell lines not containing the luciferase constructs (negative controls). Lanes labeled G/5637, GM/5637 and M/HL 60 had been loaded with the same DNA preparations with the addition of the purified plasmid preparations, which had been used for the original transfections (positive controls). The two low-molecular-weight bands appearing in almost all lanes except for the negative control lanes are derived from non-specific cross-hybridizing sequences contained in the probe.

Figure 49 Represents the inhibition of reporter activity at varying concentrations of commonly used solvents. Three solvents are tested against three cell lines.

Figure 50 illustrates the time course of bioluminescent signal decay after addition of Actinomycin D to the cell clones G1002, GM1074 and CM1. Time in hours is plotted against the logarithm of the ratio of the bioluminescent

signal generated by Actinomycin D - treated cells over the signal of untreated control cells.

Figure 51 is a quality assurance analysis of a high throughput screen measuring the ratios of negative values at various positions within a plate. The expected value is 1.0.

Figure 52 is a quality assurance analysis of a high throughput screen measuring a coefficient of variance for the negative controls on a number of plates. Values less than 10 are acceptable.

Figure 53 is a quality assurance analysis of a high throughput screen measuring a coefficient of variance for the positive controls on a number of plates. Values less than 10 are acceptable.

Figure 54 is a quality assurance analysis of a high throughput screen measuring a response of a reporter cell line to three different concentrations of a compound known to induce transcription.

Figure 55 is an analysis similar to the one shown in Figure 54 except more plates were analyzed (this is a more typical high throughput screen).

Figure 56 is a bar graph illustrating specific induction of luciferase expression in reporter cell lines for MMTV (M10), human growth hormone (532) and human G-CSF (G21) promoters in response to chemicals identified in a high throughput screen and known transcriptional inducers.

Figure 57 is a bar graph illustrating specific inhibition of luciferase expression in reporter cell lines for MMTV

(M10), human growth hormone (532), and human G-CSF (G21) in response to chemicals identified in a high throughput screen.

Figure 58 is an autoradiograph of a Northern blot illustrating increased G-CSF mRNA production by the human epithelial cell line U5637 in response to chemicals #670 and #1255 and IFN-gamma as compared to the solvent DMSO. Reprobing with beta-actin was used to normalize for the amount of mRNA that had been loaded onto the gel.

Figure 59 is an autoradiograph of a polyacrylamide gel illustrating an S1 nuclease protection analysis of increased mRNA production by the human bladder carcinoma cell line 5637 in response to lead chemicals #542, #1255, #1793 and #1904. "RNA" indicates the sources of the RNA preparations used in individual lanes. "Probe" indicates the mRNA-specificities of probes used in individual lanes. "Compound" lists the compounds with which the 5637 cells were treated prior to RNA extraction and loading on individual gel lanes ("Cyclo" means cycloheximide). "Conc" indicates three different compound concentrations used in the experiment (L = low, M = medium, H = high). G, GM and A indicate the correct sizes of G-CSF-, GM-CSF- and Actin-specific nuclease-protected mRNA/Probe hybrids.

Figure 60 illustrates a dose response analysis of chemicals #80, #670, and #1780 using the G-CSF reporter cell line G21. The amount of luciferase expression is indicated in arbitrary units.

Figure 61 is a bar graph illustrating increased G-CSF secretion by 5637 cells treated for 48 hours in serum-containing media with the samples indicated on the abscissa. TNF-alpha was used at 5 ng/ml. Chemicals #542 and

#1780 were used at 50 uM or 1 uM and 0.2 uM final concentration, respectively. Both chemicals were used in DMSO at a final concentration of 0.5 %. The ordinate indicates the concentration of G-CSF secreted into 5 ml of serum-containing media by 25 square cm of confluent 5637 cells.

Figure 62 illustrates a dose response analysis of chemical #542 using the G-CSF reporter cell line G 21 (solid line) and the MTT respiratory inhibition cytotoxicity assay (dotted line). Respiratory inhibition in percent of untreated control cells (Ordinate, left scale) and luciferase expression of #542- treated over solvent-treated cells (ordinate, right scale) are plotted against #542 concentration (abscissa).

Figure 63 represents plasmids pEPOEX3108, pEPOEX3110 and pEPOEX3112. Plasmid pEPOEX3108 contains the oxygen sensitive enhancer (A). Plasmid pEPOEX3110 contains the oxygen sensitive mRNA stability element (B) and pEPOEX3112 contains both the enhancer and the stability element (A and B).

DETAILED DESCRIPTION OF THE INVENTION

As used in this application, the following words or phrases have the meanings specified.

Antisense nucleic acid means an RNA or DNA molecule or a chemically modified RNA or DNA molecule which is complementary to a sequence present within an RNA transcript of a gene.

Directly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of the gene through the binding of a molecule to (1) the gene (2) an RNA transcript of the gene, or (3) a protein which binds to (i) such gene or RNA transcripts, or (ii) a protein which binds to such gene or RNA transcript.

A gene means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

Hematopoietic growth factor means a polypeptide capable influencing the replication of differentiation of hematopoietic progenitor cells at one or more stages of blood cell development.

Indirectly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of such gene through the action of a molecule which cause enzymatic modification of a protein which binds to (1) the gene or (2) an RNA transcript of the gene, or (3) protein which binds to (i) the gene or (ii) an RNA transcript of the gene. For example, altering the activity of a kinase which subsequently phosphorylates and alters the activity

of a transcription factor constitutes indirect transcript modulation.

Ligand means a molecule with a molecular weight of less than 5,000, which binds to a transcription factor for a gene. The binding of the ligand to the transcription factor transcriptionally modulates the expression of the gene.

Ligand binding domain of a transcription factor means the site on the transcription factor at which the ligand binds.

Modulatable transcriptional regulatory sequence of a gene means a nucleic acid sequence within the gene to which a transcription factor binds so as to transcriptionally modulate the expression of the gene.

Receptor means a transcription factor containing a ligand binding domain.

Specifically transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of such gene alone, or together with a limited number of other genes.

Transcription means a cellular process involving the interaction of an RNA polymerase with a gene which directs the expression as RNA of the structural information present in the coding sequences of the gene. The process includes, but is not limited to the following steps: (1) the transcription initiation, (2) transcript elongation, (3) transcript splicing, (4) transcript capping, (5) transcript termination, (6) transcript polyadenylation, (7) nuclear export of the transcript, (8) transcript editing, and (9) stabilizing the transcript.

Transcription factor for a gene means a cytoplasmic or nuclear protein which binds to (1) such gene, (2) an RNA transcript of such gene, or (3) a protein which binds to (i) such gene or such RNA transcript or (ii) a protein which binds to such gene or such RNA transcript, so as to thereby transcriptionally modulate expression of the gene.

Transcriptionally modulate the expression of a gene means to change the rate of transcription of such gene.

Triple helix means a helical structure resulting from the binding of one or more oligonucleotides to double stranded DNA.

The invention also provides a method for directly transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the hematopoietic growth factor encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene encoding the hematopoietic growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

In one embodiment of the method of the invention the

molecule does not naturally occur in any cell of a lower eucaryotic organism such as yeast.

In the preferred embodiment, the molecule does not naturally occur in any cell, whether of a multicellular or a unicellular organism. In a presently more preferred embodiment, the molecule is not a naturally occurring molecule, e.g. it is a chemically synthesized entity.

Preferably, the cell contacted in accordance with the method identified above is a cell from a multicellular organism, for example, an animal cell such as a rat, mouse, rabbit or human cell.

The method of the invention permits modulation of the transcription of the gene which results in either upregulation or downregulation of expression of the gene encoding the hematopietic growth factor, depending on the identity of the molecule which contacts the cell.

In one embodiment of the invention the molecule binds to a modulatable transcription sequence of the gene. For example, the molecule may bind to a promoter region upstream of the coding sequence encoding the hematopoietic growth factor.

In one embodiment of the method of the invention the molecule comprises an antisense nucleic acid which is complementary to a sequence present in a modulatable, transcriptional sequence. The molecule may also be a double-stranded nucleic acid or a nucleic acid capable of forming a triple helix with a double-stranded DNA.

In accordance with the method of this invention the hematopoietic growth factor is a colony stimulating factor,

for example, a granulocyte-macrophage colony stimulating factor, a granulocyte colony stimulating factor, or is a macrophage colony stimulating factor. In accordance with the method the hematopoietic growth factor may also be erythropoietin, IL-3, or stem cell factor. In addition, the hematopoietic growth factor may be an interleukin, a cytokine, or a lymphokine, including interferons.

The invention further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the gene encoding the hematopoietic growth factor, and (iii) a DNA sequence encoding a polypeptide other than the hematopoietic growth factor, which polypeptide is capable of producing a detectable signal. The DNA sequence is coupled to, and under the control of, the promoter, and the contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable detectable signal to be produced by the polypeptide so expressed. The amount of the signal produced is then quantitatively determined and the amount of detectable signal produced compared with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, so as to identify the molecule as one which causes a change in the detectable signal produced by the

polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

In the practice of the preceding method the polypeptide may be a luciferase, chloramphenicol acetyltransferase, glucuronidase, β galactosidase, neomycin phosphotransferase, alkaline phosphatase or guanine xanthine phosphoribosyltransferase.

The invention further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the gene encoding the hematopoietic growth factor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter. The cell is contacted under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable change in the amount of the polypeptide produced. The amount of the polypeptide produced is quantitatively determined. By comparing the amount so determined compared with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, one can thereby identify the molecule as one which causes a change in the amount of the polypeptide

expressed, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

In the foregoing methods the DNA sequence encoding the polypeptide may be inserted downstream of the promoter of the gene encoding a hematopoietic growth factor by homologous recombination.

In certain embodiments of the invention the polypeptide so produced is capable of complexing with an antibody or is capable of complexing with biotin. In this case the resulting complexes may be detected.

The invention further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of gene encoding the hematopoietic growth factor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter. The cells are contacted under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. The amount of the mRNA produced is quantitatively determined and the amount so determined compared with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample

with any other molecule so as to identify the molecule as one which causes a change in the detectable mRNA amount of, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor. In a presently preferred embodiment of the mRNA is detected by quantitative polymerase chain reaction.

In each of the preceding methods the sample comprises cells in monolayers or cells in suspension. Preferably, such cells are animal cells, or human cells. In the presently preferred method the predefined number of cells is from about 1 to about 5 X 10^5 cells, or about 2 X 10^2 to about 5 X 10^4 cells. In these methods the predetermined amount or concentration of the molecule to be tested is typically based upon the volume of the sample, or be from about 1.0 pM to about 20 μM, or from about 10 nM to about 500 μM.

Typically the contacting is effected from about 1 to about 24 hours, preferably from about 2 to about 12 hours. Also the contacting is typically effected with more than one predetermined amount of the molecule to be tested. The molecule to be tested in these methods can be a purified molecule or a homogenous sample. Further, in the method of the invention and may consist essentially of more than one modulatable transcriptional regulatory sequence.

A screening method according to any of the methods discussed above is also claimed. This screening method comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested.

In such a screening method the plurality of samples preferably comprises more that about 10^4 samples, or more preferably comprises more than about 5 X 10^4 samples.

Also provided is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding hematopoietic growth factors according to the methods discussed above.

These methods are preferably carried out with more than about 10^3 samples per week contacted with different molecules.

Pursuant to the provisions of the Budapest Treaty on the International Recognition of Deposit of Microogranisms For Purpose of Patent Procedure, the plasmid and the cell lines listed below have been deposited with the American Type Culture Collection ("ATCC"), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.:

1. a plasmid designated pUV106, deposited under ATCC Accession No. 40946;

2. a human promylelocytic cell line HL60 transfected with pCSF1-102 and pTKNeo3, designated M2086, deposited under ATCC Accession No. CRL 10641;

3. a human bladder carcinoma cell line transfected with pGUV150, designated G1002, deposited under ATCC Accession No. CRL 10660;

4. a human bladder carcinoma cell line transfected with pGMLL103neo3, designated GM1073, deposited under ATCC Accession No. CRL 10664;

5. a NIH Swiss mouse embryo cell line, NIH 3T3, transfected with the MMTV reporter plasmid, designated M10, deposited under ATCC Accession No. CRL 10659; and

6. a GC rat pituitary cell line, transfected with the growth hormone reporter plasmid, designated 532, deposited under ATCC Accession No. CRL 10663.

Further, the invention provides a method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding a hematopoietic growth factor, the expression of which is associated with a defined physiological or pathological effect in the organism, is also included. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene encoding a hematopoietic growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

In the method discussed above the molecule may bind to a modulatable transcription sequence of the gene. Also, the method discussed above includes the use of a molecule of an antisense nucleic acid, a double stranded nucleic acid, or a nucleic acid capable of forming a triple helix with double-stranded DNA.

In one embodiment of the method, the physiological effect discussed above is the protection of a hematopoietic system from damage by chemotherapeutic agent, or the protection of stem cells from damage by chemotherapeutic agents. Additionally, the pathological effect is typically a disorder where modulated expression of the gene encoding a hematopoietic growth factor is associated with amelioration of the disorder. Some of the defined pathological effects may include a hematopoietic dysfunction, tissue inflammation, atherosclerosis, viral infection, anemia, leukopenia, neutropenia, cancer, thrombocytopenia, or dysfunction in a cholesterol or other metabolic pathway.

The administering discussed in the preceeding method may comprise topical contact, oral, transdermal, intravenous, intramuscular or subcutaneous administration. Methods of administration of molecules in the practice of the invention are well known to those skilled in the art as are methods of formulating the molecule for administration depending on the specific route of administration being employed.

This invention is illustrated in the Experimental Detail section which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

A. Cell Culture

All media and reagents used for routine cell culture were purchased from Gibco (Grand Island, NY), Hazelton (Lenexa, KS), or Whittaker M.A. Biologicals (Walkersville, MD). Fetal calf serum (FCS) was from Hyclone (Logan, UT), and nutrients used for serum-free defined media were purchased from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), Bachem (Torrance, CA) and Collaborative Research (Bedford, MA). (36, 39).

A human bladder carcinoma cell line (U5637, ATCC# HTB 9) was used for transfection of plasmids containing the human Granulocyte-Colony Stimulating Factor (G-CSF), Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) and Stem Cell Factor (SCF or Steel) promoters fused to the luciferase reporter sequences (see below) and was maintained in RPMI medium supplemented with 10% FCS. For HTP screening, transfected 5637 clones were transferred to a serum free defined medium consisting of Iscove's modified Eagle's medium (IMEM) and Ham's F12 medium (1:1) supplemented with growth factors, hormones and nutrients as described previously (50), or in reduced serum (1%) medium.

A human monocyte-like promyelocytic leukemia derived cell line, HL60 (ATCC# CCL240), was used for transfection of plasmids containing the Macrophage-Colony Stimulating Factor (M-CSF) promoter. These cells were maintained in RPMI medium supplemented with 20% FCS.

A human hepatocellular carcinoma derived cell lines, Hep3B

(ATCC# HB8064) and HepG2 (ATCC# HB8065), were used for transfection of plasmids containing the Erythropoietin (EPO) promoter. These cells were maintained on MEM:OptiMEM (1:1) supplemented with 10% FCS.

A human T-cell lymphoblastic leukemia derived cell line, CCRF-HSB-2 (ATCC# CCL120.1), is used for the transfection of plasmids containing the Interleukin-3 (IL-3) promoter. These cells are maintained on DMEM medium supplemented with 10% FCS.

A murine embryonic fibroblast cell line, NIH3T3 (ATCC# CCL92), was used for the transfection of plasmids carrying the MMTV promoter. These cells were maintained on DMEM, supplemented with 10% FCS.

A rat pituitary cell line, designated GC, was used for transfection of plasmids containing the human growth hormone promoter. This cell line is maintained in DMEM and Ham's F12 medium (1:1), supplemented with 12.5% FCS.

B. Transfection

Cell were transfected by one of three methods, following manufacturer's instructions; by calcium phosphate precipitation (Pharmacia), lipofection (Life Technologies Inc.) or electroporation (BioRad). In most cases, 25-75 μg of plasmid DNA, linearized by a single restriction endonuclease cut within the vector sequences, was electroporated into approximately 5 million cells. When co-transfection of a separate neomycin resistant plasmid was employed the molar ratio of luciferase fusion plasmid to neomycin resistant plasmid was either 10:1 or 20:1. Neomycin resistant clones were selected by growth in media

containing G418 (Geneticin, Gibco).

C. Liquid Scintillation Counter Bioluminescence Assay

To assay for luciferase expression in transient expression assays in the various transfected clones, cells were incubated with various transcriptional inducers in serum free defined media, washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS, Gibco) and lysed in Lysis Buffer 1 (50 mM Tris acetate pH7.9, 1 mM EDTA, 10 mM magnesium acetate, 1 mg/ml bovine serum albumin [BSA], 0.5% Brij 58, 2 mM ATP, 100 mM dithiothreitol [DTT]). All reagents were obtained from Sigma except for DTT which was from Boehringer Mannheim. After lysis, cell debris was sedimented by brief centrifugation, and 950 µl of supernatant extract were added to a glass scintillation vial. Samples were counted individually in an LKB (Gaithersburg, MD) scintillation counter on a setting which allows measurement of individual photons by switching off the coincidence circuit. The reaction was started by addition of 50 µl of 2 mM luciferin (Sigma, St. Louis, MO or Boehringer Mannheim, Indianapolis IN) in Buffer B (Buffer B = Lysis Buffer 1 without Brij 58, ATP and DTT) to the 950 µl of lysate. Measurement was started 20 seconds after luciferin addition and continued for 1 minute. Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer.

D. Construction of the Luciferase-Fusion Reporter Vector

Unless otherwise indicated, molecular cloning procedures

were performed essentially according to Maniatis et al. (43). Oligonucleotides were synthesized by the beta-cyanoethyl phosphoramidite method according to protocols provided by the manufacturer of the DNA-synthesizer (Model 380A, Applied Biosystems (Foster City, CA).

A mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions to be used in high throughput screens to identify transcriptionally modulating chemicals. Features of the plasmid are shown in Figure 1. The shuttle vector was constructed in several steps:

The firefly luciferase gene was removed from the plant expression plasmid pDO432 (46) (Figure 2) as a 1.9 kb BamHI fragment and cloned into the BamHI site of pSVL (Pharmacia, Piscataway, NJ), a mammalian expression vector containing the SV40 promoter. The resulting plasmid (pSVLuci; Figure 3) was digested with XhoI and SalI to produce a 2.4 kb fragment containing the luciferase coding sequences and the SV40 late polyadenylation site. This fragment was inserted into the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a eukaryotic expression vector containing the MMTV promoter. The resulting MMTV promoter-luciferase fusion plasmid (pMLuci; Figure 4) was used to transfect NIH/3T3 cells as described below. Similar constructs can be made using luciferase vectors from Clontech (Palo Alto, CA).

Six oligonucleotides (pUV-1 through pUV-6) were synthesized (see Figure 5 for sequence)(SEQ ID NO: 1-6). The sequences of pUV-1, pUV-2 and pUV-3 correspond to a multicloning site, the beta-globin leader sequence and the first 53 bases of the firefly luciferase coding region. The sequences of pUV-4, pUV-5 and pUV-6 are complementary to

the first three oligonucleotides. The pUV oligonucleotides were annealed, ligated and inserted into the SalI/EcoRI sites of pTZ18R (Pharmacia, Piscataway NJ) (Figure 5). The resulting vector was then digested with SmaI/PvuII and the oligonucleotide containing fragment was cloned into the bluescript KS(+) plasmid (Stratagene, La Jolla, CA), previously digested with PvuII, to yield pUV001 (Figure 6). Several fragments were ligated into pUV001 to create pUV100: The luciferase coding sequences (except first 53 bases) and polyadenylation site were obtained as a 1.8 kilobase XbaI/XmaI fragment from pMLuci (Figure 7). The SV40 early splice site and the SV40 late polyadenylation site were obtained as an 871 bp XmaI/BamHI fragment from pMSG (Pharmacia, Piscataway NJ, Figure 7). Both DNA fragments were cloned into pUV001, previously digested with XbaI/BamHI to yield pUV100 (Figure 7).

A 476 b fragment containing a dimeric SV40 polyadenylation site was then cloned into the BclI site of pUV100 (Figure 8). To do this, a 238 bp BclI/BamHI fragment was obtained from SV40 genomic DNA (BRL), ligated, digested with BclI/BamHI, gel isolated, and inserted into pUV100, resulting in the vector pUV100-3 (Figure 8). Linkers containing one SfiI and one NotI restriction site were then cloned into the PvuII/BamHI sites of pUV100-3. Two sets of linkers were synthesized containing the SfiI site in opposite orientations (oligonucleotides D-link1 and D-link2 and oligonucleotides R-link1 and R-link2). The sequences of the oligonucleotides (SEQ ID NO: 7-10) were:

5' GATCGGCCCCTAGGGCCGCGGCCGCAT 3' (D-link1)
5' ATGCGGCCGCGGCCCTAGGGGCC 3' (D-link2)
5' GATCGGCCCTAGGGGCGGCCGCAT 3' (R-link1)
5' ATGCGGCCGCGGCCCCCTAGGGCC 3' (R-link2)

The plasmid that contains D-link oligonucleotides was named pUV102 and the plasmid that contains R-link oligonucleotides was named pUV103 (Figure 9).

The neomycin resistance gene (neo) was then placed under control of the Herpes Simplex Virus thymidine kinase (HSV-TK) promoter to generate a resistance cassette which is free of known enhancer sequences. To do this the HSV-TK promoter was synthesized using four oligonucleotides (Figure 10) (SEQ ID NO: 11-14) designed according to published sequence information (11), and including an SfiI restriction site 5' of the HSV-TK sequences. These oligonucleotides were phosphorylated, annealed, ligated and inserted into pUV100 digested previously with HindIII/NheI, generating the vector pTKL 100 (Figure 11). After verifying the HSV-TK sequence, the about 3.5 kb NheI/SmaI fragment was isolated from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region was isolated from pRSVNEO (37). These two fragments were filled in with Klenow polymerase and ligated to form pTKNEO (Figure 12). An additional SfiI site was then inserted 3' of the neo gene by isolating the about 1.8 kb SfiI/BamHI and about 2.6 kb SfiI/PVUII fragments of pTKNEO and conducting a three way ligation along with a synthesized SfiI oligonucleotide generating pTKNEO2 (Figure 13)(SEQ ID NO: 15-16). The HSV-TK/NEO vector containing an optimized Kozac sequence was also utilized (Stratagene, La Jolla, CA, pMC1NEO). An additional vector was constructed by replacing the about 0.9 kb EcoRI/SalI fragment of pTKNEO2 with the about 0.9 kb EcoRl/SalI fragment from pMC1NEO. This vector was termed pTKNEO3. (Figure 14). The SfiI fragment of pTKNEO3, containing the TK promoter and the neomycin resistance gene, was cloned into the SfiI site of pUV102 to yield pUV106 (ATCC Accession No. 40946).

E. Molecular Cloning of Hematopoietic Promoters and Insertion into the Mammalian Expression Shuttle Vector

This section describes (a) the molecular cloning of the promoter and transcriptionally modulatable regulatory sequences of the human Granulocyte Colony Stimulating Factor (G-CSF), human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), human Macrophage Colony Stimulating Factor (M-CSF), human Stem Cell Factor (SCF), human Erythropoietin (EPO) and human Interleukin-3 (IL-3) genes, and (b) the making of constructs where these regulatory sequences control the expression of the firefly luciferase gene. These constructs were transfected into cells as described above and analyzed for their utility as reporters for the discovery of gene expression modulating compounds (for example, in a high throughput pilot screen of 2,000 chemicals to identify chemicals acting as specific transcriptional modulators.

To make such constructs, several kilobases of sequence upstream of the transcription start site, along with 5' untranslated sequences up to the translation start site (ATG), of a gene of interest were inserted 5' of the luciferase coding region, along with any additional sequences (e.g. intronic enhancers) required for properly regulated expression of the luciferase reporter. In this way constructs can be made where all sequences upstream of their translation start site are from the gene of interest, and all coding sequences are from the luciferase gene. How this was accomplished for the hematopoietic growth factor genes is described below.

1. Construction of the human Granulocyte-Colony Stimulating Factor (hG-CSF) Promoter-Luciferase fusion plasmids

Information on the G-CSF upstream and coding sequences was published by Nagata et al. (45) and was used to synthesize five oligonucleotide probes (OL-1 to OL-5) to screen a human leukocyte genomic DNA library (Clontech, Palo Alto, CA) according to the supplier's instructions. The sequences of the oligonucleotide probes (SEQ ID NO: 17-21) were:

5' GCTTTTTGTTCCAACCCCCCTGCATT 3' (OL-1);
5' CCCTGCATTGTCTTGGACACCAAAT 3' (OL-2);
5' GCGCTCCAGGAGAAGCTGGTGAGT 3' (OL-3);
5' AAGCTGATGGGTGAGTGTCTTGGC 3' (OL-4);
5' ATCAGCGGCTCAGCCTTCTT 3' (OL-5);

The sequences of OL-1, OL-2 and OL-5 recognize the G-CSF promoter region, OL-4 recognizes the first intron/exon junction and OL-3 recognizes sequences within the second exon (45). One of the clones isolated from the leukocyte library using these oligonucleotide probes contains a 3.5 kb SalI-BamHI fragment of G-CSF genomic sequence consisting of 3.3 kb of promoter sequence and two hundred base pairs of the coding region. This fragment was inserted into the vector pGEM-7-Zf (Promega, Madison, WI) which had previously been digested with SalI/ BamHI, resulting in the vector pJM710 (Figure 15). pJM710 was then digested with PstI, and the resulting 1.6 kb fragment containing G-CSF upstream sequences and the first 15 bases of the G-CSF leader sequence was inserted into the PstI site of pGEM5-Luci (Figure 16) to generate pG-Lucl (Figure 17). This construct was then used for transfections of 5637 human bladder carcinoma cells as described below for the isolation of clone G21. pGEM5-Luci (Figure 16) had previously been constructed by inserting the XbaI/SalI fragment from pSVLuci (Figure 3) containing the luciferase coding sequence and the SV40 late polyadenylation signal

into pGEM 5-Zf (Promega, Madison Wl) digested with XhoI/SalI.

In order to correctly fuse the G-CSF upstream sequences to the luciferase start codon, oligonucleotides were synthesized which contain the G-CSF leader sequence from +15 to the ATG (45), and were cloned into pUC19 to create pGUC84 (Figure 18). The sequence of the inserted fragment was determined and was found to be as expected. The G-CSF-oligonucleotide-containing NcoI/ScaI fragment from pGUC84 was then isolated and ligated to the luciferase-containing NcoI/ScaI fragment from pUV100 to create pGUV100 (Figure 19). Following this, the PstI fragment of the G-CSF promoter was isolated from pJM710 (Figure 15) and inserted into the PstI site in pGUV100 generating pGUV1 (Figure 20). The rest of the G-CSF promoter clone was added by ligating the G-CSF-luciferase containing SfiI/ScaI fragment from pGUV1 to the appropriate SfiI/ScaI fragment from pJM710, creating the plasmid pGUV2 (Figure 21). The XbaI fragment from pGUV2 containing the G-CSF-luciferase fusion was then cloned into pUV103 previously digested with XbaI/SpeI, generating pGUV140 (Figure 22). Finally, a TK-Neo cassette was included by ligating the ScaI/XbaI fragment from pGUV140 which contains the G-CSF-luciferase fusion into pTKNEO103 previously digested with ScaI/XbaI, yielding the final vector pGUV150 (Figure 23).

2. Human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Cloning of GM-CSF promoter sequences was performed by using oligonucleotide probes based on the published GM-CSF genomic sequence (40). Two DNA oligonucleotide probes were synthesized, one corresponding to GM-CSF sequences 5' of

the coding region (5' GGTGACCACAAAATGCCAGGGAGGCGGG 3') (SEQ ID NO: 22) and the other to sequences in the first exon (5' GCAGGCCACAGTGCCCAAGAGACAGCAGCAGGCT 3')(SEQ ID NO: 23). The oligonucleotide probes were used to screen a human leukocyte cell genomic DNA library (Clontech, Palo Alto, CA) following the manufacturer's instructions. One clone was obtained which contains the entire GM-CSF coding region along with 2 kb of upstream sequences (see Figure 24).

The about 0.7 kb HindIII/RsaI fragment of the GM-CSF clone (Figure 24) was inserted into pUC18 previously digested with HindIII/HincII. The about 0.7 kb HindIII/SmaI fragment was then isolated from the resulting vector and cloned into pUV 100 digested previously with HindIII/SnaBI, thereby generating pGMLUCI (Figure 25). In order to correctly fuse the GM-CSF ATG with the coding region of luciferase, four oligonucleotides (Figure 26) (SEQ ID NO: 24-27) were synthesized, phosphorylated, annealed, ligated, and inserted into pUC19 previously digested with EcoRI/XbaI, generating pGM-1 (Figure 27). pGM-1 was then sequenced (Sequenase Kit, US Biochemicals, Cleveland, OH) using the M13 forward (U.S. Biochem.) and reverse primers (Pharmacia, Piscataway, NJ) to ensure that there were no mutations in the synthesized oligonucleotides. The about 1.8 kb BstEII/ScaI fragment from pGM-1 was then isolated and ligated to about 1.5 kb BstEII/ScaI fragment from pGMLUCI to generate pGM-2 (Figure 27). pGM-2 was then digested with Hind III/XbaI and about 0.7 kb fragment was cloned into pUV 102 and pUV 103 previously digested with HindIII/XbaI. This generated pGMLS102 and pGMLS103 (Figure 27), which contain 663 bp of GM-CSF sequence 5' of the ATG fused directly to the second (correct) ATG of luciferase and the rest of the luciferase coding region. An additional about 1.4 kb of upstream sequences were cloned into this construct by isolating the about 1.4 kb

SalI/HindIII fragment from the GM-CSF clone, blunting the SalI end by filling in with Klenow polymerase, and inserting the fragment into pGMLS102 and pGMSL103 previously digested with HindIII/SnaBI. This step generated pGMLL102 and PGMLL103, respectively (Figure 27). Finally, the pTKNEO2 and PTKNEO3 about 1.8 kb SfiI fragments were cloned directly into the SfiI site of pGMLL103 to generate pGMLL103 NEO2 and pGMLL103 NEO3.

3. Human Macrophage Colony Stimulating Factor (M-CSF or CSF-1)

Sequence information on the M-CSF gene (41) was used to synthesize an oligonucleotide probe (CSF1-a) to screen a human leukocyte genomic DNA library (Clontech, Palo Alto, CA) according to the supplier's instructions. The sequence of the oligonucleotide probe (SEQ ID NO: 28) was:

5' CCGGCGCGGTCATACGGGCAGCTGG 3' (CSF1-a)

The sequence of this probe corresponds to sequences within the second exon of the M-CSF gene. One of the clones isolated from the leukocyte library contains a 5 kb EcoRI/HindIII fragment which includes the first exon and 5' flanking region of M-CSF. This fragment was inserted into the pTZ18R vector (Pharmacia, Piscataway NJ) which had been previously digested with EcoRI/HindIII resulting in the vector CSF1-pTZ18 (Figure 28). The same fragment was isolated from CSF1-pTZ18, blunt ended at the EcoRI end, and inserted into the pUV100 vector which had been previously digested with SnaBI/HindIII, resulting in the vector p100-RH (Figure 29). The M-CSF untranslated leader sequence (41) was then fused to the first codon of the luciferase coding region as follows: (a) a 740 bp PstI/PvuII fragment was isolated from CSF1-pTZ18 containing

570 bp of the M-CSF promoter and 170 bp of the untranslated leader sequence; (b) oligonucleotides containing sequences from the 3' end of the M-CSF leader sequence and the 5' end of the luciferase coding region were synthesized:

5' CTGCCCGTATGGA 3' (CSF-luci5)(SEQ ID NO: 29)
5' ACGGGCAG 3' (CSF-luci6);

(c) oligonucleotides pUV3 and pUV6 (previously used to construct pUV001) were annealed, and digested with XbaI to release a 48 bp fragment which contains 48 bases of the luciferase coding region; (d) DNA fragments and oligonucleotides (from a, b and c) were ligated and inserted into pUV100 previously digested with PstI/XbaI to yield p100-2 (Figure 30). A construct containing a larger M-CSF promoter fragment (5 kb) was also made. A 2 kb XmaI fragment was isolated from the plasmid p100-2. This fragment contains the 3' end of the M-CSF leader sequence fused to the luciferase start codon. The 2 kb XmaI fragment was inserted in p100-RH previously digested with XmaI, to yield p100-RHC (Figure 31). The fused 5 kb M-CSF promoter-luciferase construct was then inserted into pUV102 as follows: a 5 kb NotI/XbaI fragment (blunt ended at the NotI end) was isolated from p100-RHC and inserted into pUV102, previously digested with SnaBI/XbaI, to generate pCSF1-102 (Figure 32). This construct was then used for transfections of HL60 promylocytic leukemia cells.

4. Erythropoietin (EPO)

Information on the EPO upstream and coding sequences has been published (42) and was used to synthesize two oligonucleotide probes (EP06 and EP08) to screen a human leukocyte genomic DNA library (Clontech, Palo Alto, CA) according to the supplier's instructions. The sequences of

the oligonucleotide probes (SEQ ID NO: 30-31) were:

5'AATGAGAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCC TGGAA 3' (EP08)

5'TTCCAGGCATAGAAATTAAC 3' (EP06)

EP08 is complementary to sequences within the third exon of the EPO gene (42). EP06 is complementary to the 3' end of EP08 and was used as a primer for filling in the complementary strand of EP08 with labelled nucleotides, thereby generating a probe for cloning. One of the clones isolated from the leukocyte genomic DNA library contained a 7.5 kb BamHI fragment consisting of 6.2 kb of the EPO promoter region and the first three EPO exons. This fragment was inserted into the plasmid Bluescript KS(+) (Stratagene, La Jolla, CA), previously digested with BamHI, resulting in the vector pEP-7.5B (Figure 33). The EPO leader sequence was fused to the start codon of the luciferase gene by using four synthetic oligonucleotides (EP09 to EP012). The sequences of the oligonucleotides (SEQ ID NO: 32-35) were:

5'CCCGGTGTGGTCACCCGGCGCGCCCCAGGTCGCTG AGGGACCCCGGCCAGGCGCGGA 3' (EP09)

5'CATCTCCGCGCCTGGCCGGGGTCCCTCAGCGACCT GGGGCGCGCCGGGTGACCACACCGGGGGGCC 3' (EP010)

5'GATGGAAGACGCCAAAAACATCAAGAAAGGCCCGG CGCCATTCTATCCT 3' (EP011)

5'CTAGAGGATAGAATGGCGCCGGGCCTTTCTTGATG TTTTTGGCGTCTTC 3' (EP012)

The sequences of EP09 and EP011 consist of 63 bases upstream of the EPO translational start site fused to the first 53 bases of the luciferase coding region. EP010 and EP012 oligonucleotides are complementary to EP09 and EP011,

respectively. These oligonucleotides were inserted into the plasmid pUV100 previously digested with ApaI/XbaI to generate the vector pRE (Figure 34). 6 kb of EPO upstream sequence was cloned into pUV102 by inserting a 6.2 kb BamHI/partial ApaI fragment from pEP-7.5 B and a 1.7 kb ApaI/XhoI fragment from pRE into pUV102 previously digested with BglI/XhoI, yielding pUV2-EP6 (Figure 35). The 1.8 kb SfiI fragment from pTKNEO3 (Figure 14) was then inserted into pUV2-EP6 previously digested with SfiI, generating pEP6.0-102-TKNEO. This construct did not produce significant luciferase activity when transfected into Hep3B cells. Analysis of the organization of the EPO expression unit indicated four alterations of the basic fusion structure were required for correct reporter expression: a. inactivation of a small (14 amino acid) open reading frame (ORF) in the 5' leader of the EPO mRNA; b. fusing the luciferase coding region to the second exon, thereby including the first intron; c. including an oxygen sensitive enhancer found at the 3' end of the EPO gene; and d. including an oxygen sensitive mRNA stability element located at the 3' end of the EPO mRNA.

The 5' ORF is inactivated using a standard oligonucleotide mutagenesis approach (59). The pEP6.0102 plasmid is digested with HinDIII and ClaI and the resulting 1990 bp fragment isolated by preparative gel electrophoresis. This fragment is then inserted into HinDIII-ClaI digested pBluescript KS(+) (Stratagene, La Jolla, California) to generate pMUT1. Single stranded DNA is generated and subsequently used for oligonucleotide mutagenesis as specified by the manufacturer of the in vitro mutagenesis kit (BioRad). The oligonucleotide utilized for mutagenesis (5' ACCGCCGAGCTTCCCGGGATCCGGGCCCCCGGTGTGGTCA 3') (SEQ ID NO: 36) changes the initiation codon of the 5' ORF (GGATGAG) to GGATCCG which simultaneously inactivates the

initiator codon and creates a BamHI site. The mutated HinDIII-ClaI fragment is then exchanged for the wild-type HinDIII-ClaI fragment of pEP6.0102 to generate pEPORF102. The neomycin resistance gene containing SfiI fragment of pTKneo3 is then ligated into the SfiI site of pEPORF102 to generate pEPORF106 (Figure 36).

The fusion of the luciferase coding region to the second EPO exon is also achieved by oligonucleotide mutagenesis. The pEP7.5B plasmid is digested with KpnI and the resulting ends trimmed off with mung bean nuclease. The mixture is then digested with HinDIII and the resulting 1.3 Kb fragment isolated by preparative gel electrophoresis. This fragment is inserted into pUV102 which has been previously digested with NheI, the overhang filled in using Klenow fragment, followed by a second restriction digest using HinDIII. The resulting plasmid (pMUT4) is used to prepare single stranded DNA for an oligonucleotide directed deletion as per the mutagenesis kit manufacturer's specifications. The oligo nucleotide used for mutagenesis (5' GTCCTGCCTGGCTGTGGCTTATGGAAGACGCCAAAAACAT 3') (SEQ ID NO: 37) fuses the luci ORF to the second exon of EPO such that the resulting chimeric protein contains 12 EPO amino acids attached to the amino terminus of the complete luciferase protein. The resulting plasmid (pMUT5) is then subjected to a second round of in vitro mutagenesis using the oligonucleotide described in the generation pEPORF102. The mutant HinDIII-Cla fragment of the resulting plasmid (pMUT6) is then exchanged with the wild-type HinDIII-ClaI fragment of pEP6.0102 to generate pEPOEX102. The neomycin resistance gene containing SfiI fragment of pTKneo3 is then ligated into the SfiI site of pEPOEX102 to generate pEPOEX106 (Figure 37).

Two oxygen sensitive regulatory elements exist in the 3'

end of the human EPO gene. On of these elements is an oxygen sensitive enhancer (60) located between bp 3282 and 3598, the other is an oxygen sensitive mRNA element located between bp 2803 and 2890 (61). The enhancer was cloned from human placental DNA by a polymerase chain reaction approach using two oligonucleotides (SEQ ID NO: 38-39) (5' CAGTCCGAGCTCCATGGGGTCCAAGTTTTG 3' and 5' CAGTAAGAGCTCAGCCCTTGCCCTGGGCAGG 3') and inserted into the SstI site in the 3' untranslated region of the EPO reporter vectors mentioned above. The RNA stability element is likewise cloned from human genomic DNA by a polymerase chain reaction and inserted into the luciferase 3' untranslated region of the EPO reporter vectors described above. EPO reporter vectors containing these two elements individually or in combination are used to create stably transfected cell lines which detect anoxia mimicking compounds which will effect either EPO transcription initiation or EPO mRNA stability or both (see Fig. 38). The vector containing the oxygen sensitive enhancer is called pEPOEX3108, the vector containing the oxygen sensitive mRNA element is called pEPOEX3110, and the vector containing both elements is called pEPOEX3112.

5. Interleukin-3 or Multi-CSF (IL-3)

Information on the IL-3 promoter and coding sequences (48, 49) was used to synthesize an oligonucleotide probe (IL-3 II) to screen a human leukocyte genomic DNA library (Clontech, Palo Alto, CA) according to the supplier's instructions. The sequence of the oligonucleotide probe (SEQ ID NO: 40) was:

5' TAAGTGTGTTATAATTTCATCGATCATGTT 3' (IL-3 II)

which corresponds to sequences within the first exon of

IL-3. One of the clones isolated from the leukocyte library using the IL-3 II probe contained an 8 kb HindII/EcoRI fragment of IL-3 sequence consisting of 6.4 kb of upstream sequences and 2 kb of the coding region. This fragment was inserted into the vector pTZ18R (Pharmacia, Piscataway NJ) previously digested with HindIII/EcoRI, resulting in the vector IL-3-pTZ18R (EcoRI-HinDIII) (Figure 38). The IL-3 leader sequence was fused to the first codon of the luciferase gene as follows. A 900 bp PstI fragment was isolated from IL-3-pTZ18R (Eco-Hind). This fragment contains 700 bp of the IL-3 promoter along with exon 1 of IL-3 (Figure 39), and was inserted into PTZ18R (Pharmacia, Piscataway, NJ) previously digested with PstI, resulting in the vector IL3/PstI-pTZ18R (Figure 39). Four oligonucleotides (IL-lu-1 to IL-lu-4) were synthesized, with the following sequences (SEQ ID NO: 41-44):

5' CCGGGGTTGTGGGCACCTTGCTGCTGCACATAT
AAGGCGGGAGGTTGTTGCCAACTCTTC 3'(IL-lu-1)

5' AGTTGGCAACAACCTCCCGCCTTATATGTGCAG
CAGCAAGGTGCCCACAACC 3' (IL-lu-2)

5' AGAGCCCCACGAAGGACCAGAACAAGACAGAGT
GCCTCCTGCCGATCCAAACATGGA 3' (IL-lu-3)

5' GTTTGGATCGGCAGGAGGCACTCTGTCTTGTTC
TGGTCCTTCGTGGGGCTCTGAAG 3' (IL-lu-4)

The sequences of IL-lu-1 and IL-lu-3 correspond to 112 bases of the 3' end of the IL-3 promoter fused to the first 5 bases of the luciferase coding region. IL-lu-2 and IL-lu-4 oligonucleotides are complementary to IL-lu-I and IL-lu-3, respectively. Oligonucleotides IL-lu-1 to IL-lu-4

along with oligonucleotides pUV-3 and pUV-6 were annealed, ligated and inserted into IL3/PstI-pTZ18R previously digested with XmaI/EcoRI, to generate pTZ-IL-3-Luci (XmaI-XbaI) (Figure 39). A 6 kb IL-3 promoter fragment was cloned into pUV102 as follows: a 500 kb NcoI/EcoRI fragment was isolated from pTZ-IL-3-Luci (XmaI/XbaI) and inserted into IL-3-pTZ18R (EcoRI/HindIII) previously digested with NcoI/EcoRI to yield pTZ-IL3-C (Figure 40). A 6.4 kb HindIII/XbaI fragment was then obtained from pTZIL3-C and inserted into pUV102 previously digested with HindIII/XbaI, resulting in the vector pUV-IL-3 (Figure 41). Finally, the 1.8 kb SfiI fragment from pTKNE03 (Figure 14) was inserted into pUV-IL-3 previously digested with SfiI, generating the vector pIL3-102-TKNEO.

6. Stem Cell Factor (SCF) or Steel (Sl)

To isolate the Stem Cell Factor (SCF) promoter, a series of three oligonucleotide probes (SEQ ID NO: 45-47) are synthesized:

Oligo 1: 5' CGCTGCGCTCGGGCTACCCAATGCGTGGAC 3'
Oligo 2: 5' AACAGCTAAACGGAGTCGCCACACCACTGT 3'
Oligo 3: 5' GCGCTGCCTTTCCTTATGAAGAAGACACAA 3'

These oligonucleotides correspond to the 5'-most region of rat-human sequence homology, the middle of the 5' leader region and the first exon of the human SCF gene, respectively (58).

A human leukocyte genomic library (Clontech, Palo Alto, California) is screened using oligos 1-3, and positive plaques subcloned into pBluescriptKS(+) (Stratagene, La Jolla, California) to create pSCF001. A set of 4 oligonucleotides (SEQ ID NO: 48-51) are designed to act as

synthetic linkers to span form the SacI site (at position -64 from the SCF AUG initiation codon) in the 5' leader of the SCF gene to the NcoI site in pUV106 (the AUG of the luciferase ORF) :

Oligo 4: 5' CCAGAACAGCTAAACGGAGTCGCCACACCACTGTTTGTGC 3'
Oligo 5: 5' AAACAGTGGTGTGGCGACTCCGTTTAGCTGTTCTGGAGCT 3'
Oligo 6: 5' TGGATCGCAGCGCTGCCTTTCCT 3'
Oligo 7: 5' CATGAGGAAAGGCAGCGCTGCGATCCAGCAC 3'

Oligo 4 is annealed to oligo 5, oligo 6 is annealed to oligo 7, and the resulting pair of linkers ligated into pGEM-5Zf(+) digested with SacI and NcoI to create pSCF002.

A SacI fragment of pSCF001, corresponding to the SCF promoter, upstream regulatory elements and a portion of the 5' leader sequence is gel purified and ligated into SacI digested pSCF002. The correct orientation of the inserted fragment is confirmed by restriction mapping to create pSCF003.

An NsiI-NcoI fragment of pSCF003, containing the entire SCF promoter and 5' leader, is gel purified and ligated into pUV102 which has been digested with PstI and NcoI to generate pSCF102. The SfiI fragment from pTKneo3 is gel purified and ligated into SfiI digested pSCF102 to generate pSCF106 (Figure 42), the SCF-luciferase reporter vector used to generate stable transfectants.

7. Human growth hormone (hGH) promoter-luciferase fusion plasmid (Used as a control in Screen I)

The SalI-XhoI fragment of pSVLuci (Figure 3) containing the luciferase coding sequences and the SV40 late polyadenylation site was inserted into pUC 8 (Biorad,

Richmond, CA), which had been linearized by a SmaI/HinCII digestion and ligated to XhoI linkers (New England Biolabs, Beverly, MA). The new plasmid thus generated (pUXLuci; Figure 43) was linearized by XhoI digestion followed by incubation with the Klenow fragment of E. coli DNA polymerase and the four deoxyribonucleotides to fill in the single-stranded ends of the vector. This linear (5.1 KB) form of pUXLuci was then ligated to the filled-in 550bP HindIII-XbaI fragment of the plasmid phGH:CAT (Figure 44) (25). Human growth hormone promoter sequences located on the HindIII-XbaI fragment were thus fused to the luciferase coding sequences located on pUXLuci generating the plasmid phGH-Luci (Figure 45), which was used in transfections of GC cells as described below (Section E2). The cell lines resulting from transfections using this vector were used for the high throughput screen as described below.

F. Isolation of Single Cell Clones Containing Various Promoter-Luciferase Fusion Constructs

1. pMluci (MMTV control cell line)

pMluci and pSV2Neo, an antibiotic resistance plasmid (47), were co-transfected into NIH/3T3 mouse fibroblast cells using the calcium phosphate precipitation method (38) with a commercially available kit (Pharmacia, Piscataway NJ). G418-resistant clones were isolated by standard methods. Once sufficient cell numbers were obtained, clones were analyzed based on several criteria: constitutive luciferase production, induction of luciferase expression by dexamethasone (1 μm, Sigma, St. Louis, MO) and acceptable standard deviation in multiple luciferase expression assays. This analysis was carried out using the luciferase

assay conditions described above. Of the clones which satisfied the above criteria for the high throughput screen, one clone, M10 (ATCC Accession No. CRL 10659), was selected for use.

2. HGH Control Cell Line

phGH-LUCI and pRSVNeo, an antibiotic resistance plasmid (14), were co-transfected into GC rat pituitary cells as described above. Selection of G418-resistant cell clones was described above except for using a concentration of 0.2 mg/ml G418. Analysis of the cell clones was performed as above, except that known inducers of hGH expression (10-100 nM) rat growth hormone releasing factor (rGRF, Bachem, Torrance, CA) and 10μm forskolin (Sigma, St. Louis, MO) were used in place of dexamethasone. One clone, 532 (ATCC Accession No. CRL 10663), was selected for further use in the high throughput screen.

3. pG-LUC1 (first G-CSF cell line)

pG-LUC1 and pRSVNeo were co-transfected using the Calcium phosphate method into 5637 human bladder carcinoma cells as described above. Analysis of G418 resistant cell clones was performed as above except that a known inducer of G-CSF expression (1-5 μg/ml lipopolysaccharide (LPS), E. coli serotype 055:b5, Difco, Detroit, MI or Sigma, St. Louis, MO) was used in place of dexamethasone. One clone, G21, was selected for use.

4. pGVU140/pTKNEO3 and pGVU150 (second and third generation G-CSF cell lines

U5637 bladder carcinoma cells were transfected with pGVU150 or pGVU140 plus pTKNeo3 either by electroporation using a BRL (Gaithersburg, Maryland) Cellporator electroporation device or by lipofection using BRL lipofectin and following the manufacturer's protocol. Neomycin resistant clones were analyzed for luciferase expression and those testing positive expanded further and frozen in liquid nitrogen. Further analysis of 6 clones (G1002, G2005, G2071, G2085, G3014, G3031) included Southern blotting, reaction to known inducers, satisfactory attachment to microtiter plates and acceptable standard deviation in multiple luciferase expression assays. Clone G1002 (ATCC Accession No. CRL 10660) was selected for use in the high throughput screen.

5. pGMLL103 NEO3 (GM-CSF)

Six clones generated by electroporation of U5637 cells with pGMLL103 NEO3 were subjected to further analysis as described above: GM1073 (10 ug; circular); GM1081, GM1088 and GM1090 (5 ug; linear); and GM1098 and GM1105 (10 ug; linear). Clone GM1073 (ATCC Accession No. CRL 10664) was selected for use in the high throughput screen.

6. pCSF1-102/pTKNeo3 (M-CSF)

Three clones generated by co-electroporation of HL60 cells with linearized pCSF1-102 and linearized pTKNeo3 were subjected to further analysis as outlined above: M2071, M2085 and M2086. M2086 (ATCC Accession No. CRL 10641) was selected for use in the high throughput screen.

7. pEPOEX106 (Epo)

Hep3B cells are electroporated with 75 ug of linear pEPOEX106. Neomycin resistant clones are isolated and subjected to the analyses outlined above (luciferase expression, Southern blot, induction by cobalt chloride, etc.), and the best clone selected for use in the high throughput screen.

8. pIL3-102-Neo3 (IL-3)

CCRF-HSB-2 cells are electroporated with 75 ug of linear pIL3-120-Neo3. G418 resistant colones are isolated by growth in G418 containing soft agar. Resistant clones are subjected to the analysis outlined above. Induction by PMA/PHA treatment is tested, and the best clone selected for use in the high throughput screen.

9. pSCF106 (SCF)

U5637 cells are electroporated with 75 ug of linear pSCF106. Neomycin resistant colones are isolated and analyzed as outlined above. Repression by Interleukin-1 is tested and the best clone selected for use in the high throughput screen.

G. Isolation of Total Cellular RNA

Total cellular RNA was isolated from the luciferase-fusion containing cell clones or from untransfected host cells following incubation with various transcriptionally

modulating chemicals identified in the high throughput screen or known previously to affect gene expression. Cells were grown in serum free medium as described above. Total cellular RNA was isolated using the RNAZol method (CINNA/BIOTECX, Friendswood, TX, Laboratories International, Inc.). Cells were resuspended and lysed with RNAZol solution (1.5 ml/9 cm petri dish) and the RNA was solubilized by passing the lysate a few times through a pipette. Chloroform was added to the homogenate (0.1 ml/ml), and samples were shaken for 15 seconds followed by a 5 minute incubation on ice. After centrifuging for 10 minutes, the upper phase was collected and an equal volume of isopropanol was added. Samples were incubated for 45 minutes at -20°C, and the RNA was pelleted for 15 minutes at 12,000 x g at 4°C. The RNA pellet was then washed with 70% ethanol and dried briefly under vacuum.

H. Northern Blotting

Total cellular RNA was isolated from cells following incubation with chemicals as described above and electrophoresed in a 1% Agarose Formaldehyde gel. The RNA was transferred to Duralon-UV nylon filters (Stratagene, La Jolla, CA) using the manufacturer's recommended protocol. The filters were prehybridized for 4 hours (prehybridizing solution = 5X SSC, 50 mM sodium pyrophosphate, 10X Denhardt's solution, 10% dextran sulfate, 7% SDS and 250 μg/ml denatured ssDNA) and then hybridized in the same solution for 16 hours at 65°C in the presence of specific probes. The G-CSF probe was a 0.6 kb AflII to XhoI fragment which contained most of exon 5 of the human G-CSF gene. The β-actin (Oncor, Gaithersburg, MD) probe was used as a control probe to normalize for the total amount of RNA. The probes were labeled with alpha-32P dCTP using a random primed DNA labeling kit (Amersham, Arlington, IL).

Following hybridization, filters were washed three times at room temperature with 1X SSC, 0.13% SDS and three times at 65°C with 0.2X SSC, 0.1% SDS. Filters were first probed with the G-CSF specific probe and then reprobed with β-actin-probe. Exposure to x-ray film was performed overnight. Bands were excised and counted in a liquid scintillation counter (LKB, Gaithersburg, MD), and counts obtained with the G-CSF specific probe were normalized relative to the counts obtained with the β-Actin specific probe.

I. S1 Nuclease protection

S1 Nuclease protection assays were carried out as described in reference 118.

J. Southern blotting

To monitor correct and complete stable integration of transfected promoter/reporter constructs, stably transfected cell clones were subjected to Southern blot analysis (51). Genomic DNA was prepared of each clone to be tested and restriction endonuclease digested with Dra I or another suitable enzyme. After electrophoresis, transfer to nylon filters and immobilization by UV irradiation using a Stratalinker UV device (Stratagene, La Jolla, California), integrated promoter/luciferase fusion constructs were visualized by probing with radioactively labelled XbaI-EcoRI fragments of the luciferase coding region. Probes were labelled using the random primer method (52). Since Dra I cuts in the SV40 polyadenylation sites located in the mammalian expression shuttle vector just upstream the inserted promoter sequences as well as downstream of the luciferase coding region, but not in any of the promoter sequences used for generating stably transfected cell

clones which were analyzed in this way, a single fragment should be visualized by the probe used. The size of that fragment should be characteristic for each of the promoter sequences analyzed.

5

K. Polymerase Chain Reaction

Total RNA was isolated using the approach described above, first strand cDNA generated by either oligo dT, random
10 hexamer or gene specific oligonucleotide priming. Specific amplification oligonucleotides were added, and the polymerase chain reaction carried out according to established methods (56).

15 RNA levels are quantitated by established methods (57) which include the addition of varying amounts of a control RNA and thereby establishing a standard curve. PCR products are visualized on an ethidium bromide stained agarose gel and are quantitated by measuring the
20 incorporation of radiolabelled deoxynucleotide triphosphates using liquid scintillation.

L. High Throughput Quantitative PCR

25 Current methods for measuring changes in gene expression suffer from various limitations. Conventional direct analysis of changes in mRNA levels (nuclease protection, Northern blot, primer extension) lack sufficient sensitivity for use with high throughput formats (e.g. 96
30 well plate cell culture). These methods also require difficult analytical procedures (e.g. sequencing gels) complicating automation. The use of gene fusions (luciferase or CAT transcriptional fusions) as demonstrated above, provide sufficient sensitivity and ease of analysis
35 but require disruption of the native transcription unit and

loss of chromosomal context, leading to potential artifact. This section of the invention proposes to circumvent the sensitivity limitation of direct analysis by using the amplification potential inherent in the polymerase chain reaction. Combining PCR with the ease of florescence detection of will allow direct mRNA analysis in a high throughput mode.

The following description outlines a high throughput drug screen utilizing direct PCR quantitation of mRNA in its most simple format.

1. Grow cells. Cells are grown in 96 well microtiter plates as described above. The final detection step is a fluorescence measurement, so an opaque (non-reflecting black) plate is required.

2. Add compounds. As with the luciferase reporter screen, compounds are added at several concentrations and at several replications. The number of duplicate samples required can be determined statistically after the basic assay is formatted (currently, quadruplicates are required).

3. Incubate. The incubation time depends on the biology of the systems studied. As with the current luciferase reporter assay, the incubation time is 6 hours.

4. Lyse cells: The cells are lysed in a buffer which satisfies several important criteria. A. Avoidance of extremes of temperature. B. Complete inactivation of contaminating cellular nucleases. C. Compatible with subsequent RNA purification steps. D. Rapid and efficient lysis. Chaotropic buffers have been

described which satisfy these requirements. Guanidine HCl (6M) will efficiently lyse cells and effectively inactivate cellular nucleases. The kinetics of nucleic acid hybridization are largely unaffected by these conditions. Thus, the subsequent RNA purification (separation using magnetic oligo dT beads, see below) does not require a buffer change.

5. Add external control: The exponential nature of the PCR amplification step tends to magnify small differences in conditions. Key to the usefulness of this approach is the careful inclusion of appropriate standards for control purposes. An artificial polyadenylated RNA (generated with phage T7 RNA polymerase and commercially available vectors, Promega Madison WI) is added at this point. This RNA serves as a control for all of the following steps: purification, cDNA synthesis, PCR amplification, PCR product purification and detection. This control RNA is added to several lysates at varying concentrations, generating a standard curve.

6. Purify RNA: RNA is purified using commercially available oligo-dT tagged magnetic beads. These beads are added to the lysate, allowed to hybridize to the mRNA, brought to the bottom of the plate using a strong magnet, and extensively washed to remove protein and DNA.

7. Synthesize cDNA: cDNA is generated using the 3' end of the bead bound oligo-dT.

8. Add PCR primers: Each gene (or control) to be assayed requires two oligos. The pair are designed so that they span a large intron. This makes the

amplification much more RNA specific. The short, spliced RNA target is much more efficiently amplified than the longer, contaminating genomic DNA target. One of the oligo pair is tagged at its 5' end with a fluorescent label. The other oligo is tagged at its 5' end with biotin (for future purification, see below). Several sets of oligos are added to each lysate. A set for each control and a set for each gene to be assayed. Every set has a different fluorescent tag.

9. PCR Amplification: Simultaneous incubation of many plates is frequently required, so either a large array of blocks or a large capacity convection oven is necessary.

10. Purification of PCR products: The PCR products are separated from the unreacted oligos using a method similar to the one employed for the initial RNA purification. Magnetic beads, tagged with streptavidan are added to the mixture. PCR products are tagged on one end with biotin (on the other with a fluorescent label) and tightly attach to the magnetic beads. The beads, along with the labeled oligonucleotides, are brought to the bottom of the plate with a magnet and extensively washed. Alternatively, fluorescently tagged PCR products are resolved electrophoretically and quantitated with a scanning gel fluorimeter (Applied Biosystems).

11. Detection: The plates are read in a 96 well fluorimeter (Ammersham).

12. Data analysis: A ratio of fluorescence from a particular gene's PCR product to the signal from the

"constitutive" internal control gives the relative mRNA level. Changes in this ratio indicates a change in gene expression. Absolute mRNA levels are determined by control experiments using carefully quantitated artificial RNAs to construct standard curves for each gene studied. This establishes a given ratio (to the internal control) for a given cellular RNA concentration.

M. PCR mRNA Detection and Quantitation of Hematopoietic Growth Factor Genes

Oligonucleotides were designed for the specific detection of each of the hematopoietic growth factor genes to be analyzed (SEQ ID NO: 52-63).

G-CSF	5'TGGCGCAGCGCTCCAGGAGAAGCTG3'	and
	5'CGCTATGGAGTTGGCTCAAGCAGCCTGC3'	
GM-CSF	5'GAGTAGAGACACTGCTGCTGAGATG3'	and
	5'GGCGGGTCTGTAGGCAGGTCGGCTC3'	
M-CSF	5'CTCCAGCCCGCAGCTCCAGGAGTCTG3'	and
	5'CCCTCTACACTGGCAGTTCCACCTG3'	
EPO	5'GGCCAAGGAGGCCGAGAATATCACG3'	and
	5'GCCAGACTTCTACGGCCTGCTGCCCGAC3'	
IL-3	5'TCAGCAATTGAGAGCATTCTTAAA3'	and
	5'GTCCTTGATATGGATTGGATGTCG3'	
SCF	5'ACTAATAATGTAAAAGACGTCACTAAATTG3'	and
	5'TCTCGCTTATCCAACAATGACTTGG3'	

In each case, these oligonucleotides were chosen to amplify sequences which span two intron splice junctions and one exon in order to minimize the nonspecific signal generated by contaminating genomic DNA (57). The oligos for G-CSF, EPO and SCF are complementary to regions within their respective gene's exons II and IV and amplify the region corresponding to exon III (42, 45, 58). The oligos for GM-CSF are complementary to regions of hGM-CSF exons I and III and amplify the region corresponding to exon II (40). The oligonucleotides for IL-3 are complementary to regions of hIL-3 exons III and V, and amplify the region corresponding to exon IV (49). The oligonucleotides for M-CSF are complementary to regions of hM-CSF exons VI and VIII and amplify the region corresponding to exon VII (41).

To test the quantitative ability of the polymerase chain reaction, total RNA was isolated form U5637 bladder carcinoma cells and diluted in two-fold serial steps to yield samples ranging from 4 to 0.05 micrograms. These RNA samples were used to generate cDNA using random primers (57) and then mixed with a constant amount of phage lambda DNA (0.2 ng) as a control for amplification efficiency. Alpha-32P-dATP was included in the PCR buffer to allow quantitation of the amplified products. The M-CSF specific PCR oligonucleotides describe above were added to the standard reaction mixture (2 pmoles per sample) and PCR carried out for 35 cycles in a Perkin-Elmer-Cetus thermal cycler. The products of the reaction were electrophoresed on a 3% NuSieve agarose gel. The gel was dried and used to expose Kodak X-OMAT AR film. The resulting autoradiogram is shown in Figure 46. This autoradiogram was quantitated using an LKB laser densitometer, the data are shown in Figure 47. The graph plots the amount of M-CSF specific product divided by the constant lambda DNA signal. The reaction was clearly quantitative for the RNA samples

between 0.05 and 1 microgram (total RNA), and proved to be a very sensitive assay for M-CSF mRNA, which is barely detectable in this cell line by conventional S1 analysis (not shown).

N. Validation of the Hematopoietic Growth Factor Reporter Cell Lines

Cell clones transfected with the OSI mammalian expression shuttle vector fused to the G-CSF, GM-CSF and M-CSF promoters were analyzed for correct and complete integration of the promoter/luciferase constructs by Southern blotting (see materials and methods).

As shown in Figure 48, G-CSF clones G1002, G3014 and G3031, GM-CSF clones GM1073, GM1088 and GM1105 and all 3 M-CSF clones tested contained only a single fragment with the correct molecular size (uppermost fragments in plasmid controls G/5637, M/HL60 and GM/5637 generated by loading mixtures of the purified plasmids and extracts of the parental cell lines 5637 or HL60 on the gel). The 2 smaller fragments are non-specific, cross-hybridizing probe impurities. The other clones all contained additional rearranged fragments of various molecular sizes. Conspicuously, all 3 G-CSF clones with correctly integrated promoter/reporter constructs were derived from electroporation, whereas the other G-CSF cell clones analyzed were obtained either by lipofection (G2005, G2071 and G2085) or calcium phosphate precipitation (G21; see Materials and Methods). G21 cells contain multiple copies of the promoter/luciferase construct, the majority of which migrate at approximately correct molecular weight. The data suggest that electroporation under the optimized conditions described is the transfection method of choice to obtain cell clones with correctly integrated, complete

promoter/luciferase reporter constructs.

Cell clones with correctly integrated promoter/reporter constructs were analyzed for correct reaction to known transcriptional inducers. Of the G-CSF clones tested, G1002 showed the most consistent levels of induction after 10.5 hours of incubation in serum-containing media with 8.3 ng/ml tumor necrosis factor-alpha (TNF-alpha; 2 fold), 20 ng/ml phorbol-myristate-acetate (PMA; 4.4 fold), 0.5 ng/ml Interleukin-1 beta (2.6 fold), a mixture of 4.2 ng/ml TNF-alpha and 0.3 ng/ml Interleukin-1 beta (3.8 fold) and a mixture of 4.2 ng/ml TNF-alpha and 10 ng/ml PMA (7.6 fold). Both TNF-alpha and PMA induction levels of clone G1002 were influenced by the presence or absence of epidermal growth factor (EGF). 7-Hour incubations in serum-free defined media with 20 ng/ml EGF resulted in a 3 fold G-CSF promoter induction by TNF-alpha versus 4 fold in the absence of EGF. The 9.3 fold induction by PMA in the absence of EGF was reduced to 5.6 fold by including EGF in the serum-free incubation mixture. Similar differences were observed, when EGF was substituted by 10% fetal calf serum.

A 7-hour incubation of G21 cells with PMA in serum-free media increased luciferase expression directed by the G-CSF promoter by 34.6 fold in the absence and by 24.6 fold in the presence of EGF. TNF-Alpha induction did not significantly change on EGF addition (2.8 fold with and 2.1 fold without EGF).

Promoter induction experiments were also conducted with the GM-CSF reporter cell lines GM1073, GM1088 and GM1105, which were all shown to contain correctly inserted constructs (see above). 10.5 hours incubation of GM 1073 cells with 20 ng/ml PMA in serum-containing media resulted in a 3.4 fold induction of the GM-CSF promoter, which was increased to

7.5 fold in serum-free media. Luciferase expression of clones GM1088 and GM1105 was induced by PMA 2.8 fold and 2 fold, respectively, while TNF-alpha induced both clones 2 fold.

All three M-CSF clones responded to a 16-hour incubation with 2,000 units/ml Interferon-gamma by a 20-fold increase of luciferase expression from the M-CSF promoter.

All 3 GM-CSF clones described above attached to the well surfaces of microtiter plates after overnight incubation. Levels of luciferase expression from clone 1105 were not appreciably affected by the omission of the fibronectin-coating step before cell plating. Luciferase expression levels were strongly increased by fibronectin coating, however, when clones G1002 or G21 were used (about 8 fold or 3 fold, respectively).

Clones GM1088, GM1073 and G1002 consistently produced bioluminescence signals varying by less than 10 % between wells, when multiple 96-well microtiter plates containing these cells were assayed.

O. Solvent Control

To be able to screen large random collections of compounds it may be necessary to use a variety of solvents to account for different solubilities of the compounds of interest. The effect of three organic solvents, DMSO, methanol and ethanol, which are frequently used to dissolve screening samples, was therefore determined on three reporter cell lines.

Cells of the lines G2005, GM1105 and M10 containing luciferase reporter constructs for the G-CSF, GM-CSF or the

promoter of the mouse mammary tumor virus were seeded into 96 well microtiter plates (10,000 cells/well) and cultured overnight. Various amounts of DMSO, methanol or ethanol were added to cultures. Luciferase activity in the cells was determined 8 hours after addition of the solvents. The relative amount of luciferase activity compared to untreated controls is plotted versus solvent concentration (Figure 49).

G2005 and GM1105, which were constructed using the same parental cell line show very similar behavior. A final concentration of 1% can be used in each case.

P. High Throughput (HTP) Screening

Cell plating: Dynatech Microlite 96 well plates were pretreated for cell attachment by Dynatech Laboratories, Inc.(Chantilly, VA). Alternatively, the 96 well plates were treated with 50 µl per well of human fibronectin (hFN, 15 µg/ml in PBS, Collaborative Research, Bedford, MA) overnight at 37°C. hFN-treated plates were washed with PBS using an Ultrawash 2 Microplate Washer (Dynatech Labs), to remove excess hFN prior to cell plating. M10 and G21 cells maintained in their respective serum media (with 0.2 mg/ml G418) were washed with PBS, harvested by trypsinization, and counted using a hemocytometer and the Trypan Blue exclusion method according to protocols provided by Sigma, St. Louis, MO Chemical Company. Cells were then diluted into serum free defined media (with 0.2 mg/ml G418), and 0.2 ml of cell suspension per well was plated onto Dynatech treated plates (G21) or hFN-treated plates (M10 and 532) using a Cetus Pro/Pette (Cetus, Emeryville CA). Plates were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere.

Addition of Chemicals to Cells: Chemicals were dissolved in DMSO at concentrations of 3-30 mg/ml. A liquid handling laboratory work station (RSP 5052, Tecan U.S. Chapel Hill, NC) was used to dilute the chemicals (three dilutions; 5 fold, 110 fold, and 726 fold). 10 µl of each dilution were added to each of quadruplicate samples of cells contained in the wells of 96-well Dynatech Microlite Plates. Cell plates were then shaken on a microplate shaker (Dynatech, medium setting, 30 sec.) and incubated for 6 hours at 37 oC, 5% CO_2.

Bioluminescence Assay: After incubation with OSI-file chemicals, cell plates were washed 3 times with PBS using an Ultrawash 2 Microplate Washer (Dynatech Labs) and 75 µl of Lysis Buffer 2 were added to each well (Lysis Buffer 2 is the same as Lysis buffer 1 except that the ATP and DTT concentrations were changed to 2.67 mM and 133 mM, respectively). Bioluminescence was initiated by the addition of 25 µl 0.4 µM Luciferin in Buffer B to each well, and was measured in a Dynatech ML 1000 luminometer following a 1 minute incubation at room temperature. Data were captured and analyzed using Lotus-Measure (Lotus) software.

More recently the cell lysis buffer was modified to also contain the luciferin. Therefore, lysis of cells and the bioluminescence reaction begin simultaneously and the production of bioluminescent light reaches a maximum at about 5 min. The level of light output declines by about 20% within further 30 min. For better lysis buffer stability bovine serum albumin has been omitted. This improved lysis buffer has been shown to remain fully functional for at least 12 hours, when kept on ice and protected from direct light.

Also, more recently, a fully automated device as described in U.S. patent application #382,483 was used to incubate luciferase reporter cells in 96-well microtiter plates, transfer chemicals and known transcriptional modulators to the cells, incubate cells with the chemicals, remove the chemicals by washing with PBS, add lysis buffer to the cells and measure the bioluminescence produced.

An additional recent improvement is the ability to screen suspension cell lines in the automated high throughput mode using opaque, 96 well filter plates (Millititer Plates, Millipore Corp.). This involved the manufacture of a robotic filtration and washing station.

Q. MTT Cell toxicity assay

To determine cytotoxic concentrations of chemicals registering as positives in the high throughput luciferase assay the MTT cytotoxicity assay was employed (54). In this assay, a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT] is reduced to a colored formazon product by reducing enzymes present only in living metabolically active cells.

R. Two-antibody Sandwich Immunoassay

Supernatants from 5637 bladder carcinoma cells incubated with chemicals registering as positives in the G-CSF promoter/luciferase high throughput assay were assayed for secreted G-CSF protein using the two-antibody sandwich immunoassay (55). The G-CSF Assay kit manufactured by Oncogene Science, Inc. was used and the manufacturer's instructions were followed.

RESULTS

A. In vivo signal half-life of the luciferase reporter system

When screening for inhibitors rather than inducers of transcription, the half-life of the reporter molecule becomes a crucial parameter in determining the minimal incubation time that would be necessary to allow enough decay of reporter signal so that the inhibition of their synthesis became detectable. The cell lines G1002 and GM1074 containing luciferase reporter constructs for the G-CSF or GM-CSF were therefore tested for the time dependency of luciferase activity after treatment of the cells with Actinomycin D, an inhibitor of transcription. This experiment measures the combined half-life of luciferase mRNA and of the luciferase protein.

Cells derived from clones G1002 and GM1074 were seeded into 96-well microtiter plates (20,000 cells G1002 or GM1074/well) and incubated overnight in cell culture conditions. At time 0 Actinomycin D (25 µg/ml) was added. At the times indicated in Figure 50 cells were washed with PBS and luciferase activity of Actinomycin-treated cells determined as described in Materials and Methods. The logarithm of the treated/untreated ratio is plotted versus time.

As demonstrated in Figure 50, apparent half-lives found in the two cell lines tested ranged from about 2.5 to 6 hours. A 24 hour incubation with a 100% efficient inhibitor of transcription would therefore be sufficient to reduce luciferase levels to maximal 6% of the control in the tested cell lines.

B. Quality Assurance Analysis of High Throughput Screens

A number of quality assurance criteria are routinely assessed during the course of high throughput screens. Data from QA analysis of a portion of Screen III (see below) are shown in Figures 51-55. Figure 51 shows an analysis of the consistency of the luciferase signal on various areas of the plate. The ratios of negative control values from three different areas within each plate are calculated and plotted versus plate number. The expected value is 1.0. Values greater than 1.2 or less than 0.8 indicate uneven signal generation across the plate. In this example 48 plates, representing 98 compounds, tested against three cell lines, are shown. The coefficient of variance for the 12 negative control values from each of the same 48 plates are represented by the data shown in Figure 52. Values less than 20% are considered acceptable. Similar data for the 12 positive control values of the same plates are shown in Figure 53. Figure 54 shows the transcription induction ratio (TIR) for the positive controls of one cell line represented in the same set of 48 plates. The TIR is the ratio of the experimental values to the untreated controls. In this case the cell line is the MMTV reporter and the positive control is dexamethasone. Three values are shown for each plot, representing three different concentrations of dexamethasone. The expected value for such an analysis depends on the promoter and inducer, but for this combination, typical values range from 20 to 40 fold. Figure 55 is an analysis similar to that shown in Figure 54 except that it is for a larger, more typical batch (190 plates). The minima observed at plates 61, 64 and 100 resulted from mechanical failure of the robotic reagent addition station, clearly demonstrating the usefulness of such quality assurance analysis.

C. High-Throughput Drug Screen

1. Screen 1

Table 1 shows a summary of the results of a one-week, high-throughput screen of 2,000 chemicals to identify those chemicals specifically stimulating or inhibiting transcription from the G-CSF, MMTV or human growth hormone (as a control for specificity) promoters. This screen concurrently tested chemicals at three concentrations on quadruplicate samples of the M10, 532 and G21 cell lines. A minimum stimulation of one promoter, to the degree indicated, and less than 50% activation of the other promoter was required for a chemical to be considered a selective activator. A minimum inhibition of 3 fold of one promoter and less than 20% inhibition of the other promoter was required for a chemical to be considered a selective inhibitor. The data are summarized in table 1. Figure 56 illustrates the transcriptional stimulation and Figure 57 the transcriptional inhibition observed with some of the lead chemicals. Lead chemicals are identified in table 2.

TABLE 1

SUMMARY OF HIGH-THROUGHPUT SCREEN I

Number (%) of Chemicals Which Activate Expression:

	2-3X	3-5X	5-7X	7-10X	>10X	Total
G-CSF	NA	23 (1.1%)	10 (0.5%)	3 (0.15%)	2 (0.10%)	38 (1.9%)
MMTV	15 (0.7%)	1 (0.05%)	0 (0%)	1 (0.05%)	1 (0.05%)	18 (0.9%)
hGH	NA	NA	12 (0.6%)	5 (0.03%)	6 (0.03%)	23 (1.14%)

Number (%) of Chemicals Which Inhibit Expression >3 Fold

Promoter		
G-CSF	7	(0.35%)
MMTV	1	(0.05%)
hGH	42	(2.1 %)

TABLE 2

A) TRANSCRIPTIONAL ACTIVATORS

Chemical#	Chemical Name	FOLD INDUCTION RELATIVE TO SOLVENT CONTROL GCSF	hGH	MMTV
G-CSF:				
40	3-Acetyl-2-6-Bis(tertiary butyl amino)-4-methyl-pyridine	5.62	0.62	0.27
58	1-Acetylimidazole	6.03	0.17	0.42
237	N-Carbethoxy-phthalimide	4.77	0.06	0.62
254	1-(2-Chloroethyl)piperidine	4.09	0.90	0.98
364	Melamine	3.67	1.18	1.07
473	1,3,5,-Triazine	>3	0.50	0.87
542	5-Bromo-2'-deoxycytidine	6.28	1.08	1.26
543	5-Bromo-2'-deoxyuridine	7.17	0.72	0.98
878	Blueberry leaf extract	3.84	1.17	0.78
1025	Culvers Root extract	4.09	0.98	1.24
1234	4-Aminocinnamic Acid hydrochloride	4.97	0.51	1.03
1255	1-Bromo-3,5-dichlorobenzene	6.74	0.43	1.09
1374	4'-Amino-N-methylacetanilide	11.03	0.05	1.05
1375	4'-(aminomethyl)benzene sulfonamide hydrochloride	8.94	0.04	1.37
1376	2-Amino-5-Methyl benzene sulfonic acid	6.37	0.04	1.32
1397	5-Amino-3-methylisothiazole hydrochloride	3.63	0.57	1.13
1482	2-Aminophenyl disulfide	3.99	0.54	1.07
1483	4-Aminophenyl disulfide	4.64	0.38	1.09

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1521	2-Amino-6-purinethiol	3.59	0.73	0.92
1583	8-Bromoadenosine	5.82	0.12	0.88
1592	Bis(2,2,3,3,4,4,5,5,6,6,7,7,) dodecafluoroheptyl-(+)-camphorate	3.20	0.74	1.34
1783	Cupferron	6.55	0.32	0.89
1793	Cyanomethyl-N,N-dimethyl dithiocarbamate	9.50	0.52	1.21
1994	3-Bromobiphenyl	3.29	0.34	0.63
2001	1-Bromo-4-tertiary butyl benzene	3.11	0.74	1.12
2030	4-Bromo-2-fluoro-6-nitroanizol	5.53	0.67	0.87
2096	(+)-1-Bromo-3-Chloro-2methyl propane	3.27	0.61	0.89
2097	1-Bromo-5-Chloro pentane	5.09	0.88	1.22
2129	4-Chlorobenzyl Chloride	3.23	0.75	0.95
GROUP A:				
378	7-Oxo-7H-benzo[e]pyrimidine 4-carboxylic acid	4.12	0.26	0.59
423	Quinacrine dihydrochloride hydrate	2.39	0.56	0.64
427	Resazurin	3.14	0.43	0.71
836	Thionin	3.20	0.23	0.58
1776	Cresyl Violet Acetate	3.50	0.15	1.36
1904	9-Aminoacridine hydrochloride	4.12	0.54	0.82
GROUP B:				
670	Methyl Green	>3	0.52	0.79
1780	Crystal Violet	20.39	0.38	1.15

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
GROUP A AND B:				
80	Acridine Orange	5.87	0.66	0.83
hGH:				
70	2-Acetylpyrrole	0.43	9.26	0.85
299	10,11-Dihydrocarbamazepine	0.53	5.46	0.47
322	1-ethyl-2-benzimidazolinone	0.60	11.18	1.12
325	Fisetin	0.14	5.42	1.0
552	3-(4-chlorophenyl)-1-methoxy-1-methyl urea	0.81	5.31	0.86
790	Rivanol	0.01	5.94	0.58
792	Rose Bengal	0.94	5.31	1.21
856	Tripaμmitin	0.28	6.49	0.42
1004	Arnica 4x	0.85	6.48	1.22
1160	Rochester # 6180	0.38	5.79	0.80
1251	Bromocresol Green	0.14	15.19	0.33
1337	4-Amino-5-hydroxy-1-naphthalene sulfonic acid	0.07	15.87	0.23
1499	2-Amino-4-phenylthiazole hydrobromide monohydrate	0.24	5.55	0.61
1550	2-Aminothiazole	0.04	5.44	0.87
1552	2-amino-2-thiazoline	1.23	7.26	0.52
1561	4-Amino-3,5,6-trichloropicolinic acid	0.23	8.05	0.48
1598	N,N'-Bis-[3-(4,5-dihydro-1H-imidizol-2-yl)phenyl] urea dipropanoate	0.72	5.32	1.27

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1678	4,8-Bis(hydroxymethyl)-tricyclo [5,2,1,$0^{2,6}$]decane	0.36	7.08	0.89
1740	5-carbethoxy-2-thiouracil	0.74	17.77	0.87
1747	N_6-carbobenzyloxy-L-lysine	0.78	6.16	0.86
1804	Cyclobutane carboxylic acid	1.05	9.41	0.49
1876	Alec Blue	0.87	11.91	0.40
1881	Alizarin Blue Black B	0.21	18.87	0.69
MMTV:				
189	Bathocuproinedisulfonic Acid disodium salt hydrate	1.06	1.47	2.80
453	2,2':6',2"-Terpyridine	0.79	0.58	13.30
519	b-Apo-8'-carotenal	1.15	0.68	2.76
562	Copaiva Balsam	1.10	0.15	2.34
629	Homoveratric acid	0.85	1.05	2.48
633	5-Iodorotic acid	1.02	0.86	2.46
765	Prednisolone-21-Acetate	0.96	1.30	2.66
828	2,4,5,4'-Tetrachlorodiphenylsulfide	1.47	1.34	2.20
848	Triamcinolone acetonide	0.75	1.28	2.43
944	Peanut	1.15	0.91	2.10
1269	5-Amino-4,6-dichloropyrimidine	0.72	0.91	2.18
1316	2-Aminofluorene	0.74	1.39	2.33
1318	2-Amino-9-fluorenone	1.13	0.85	2.41
1384	2-Amino-4'-methylbenzophenone	1.33	0.50	2.43

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1573	5-Bromoacenapthene	1.49	0.34	4.30
2064	4-(Bromomethyl)-6,7-dimethoxy-coumarin	0.82	1.10	2.53
2148	2-chlorocyclohexanone	0.45	0.92	2.82
2191	Chloramphenicol	0.37	0.35	7.32

B) TRANSCRIPTIONAL INHIBITORS

Chemical#	Chemical Name	FOLD INHIBITION RELATIVE TO SOLVENT CONTROL GCSF	hGH	MMTV
G-CSF:				
209	4-Benzoylpyridine	6.66	1.08	0.81
371	Morin hydrate	11.11	0.41	0.89
660	Maclurin	10.0	0.34	1.04
798	Salicylamide	4.76	0.90	0.68
2009	4-Bromo-3,5-dimethylpyrazole	3.70	0.57	0.64
2082	4-Bromo-3-Methylpyrazole	5.26	0.65	1.23
2121	3-Chlorobenzyl alcohol	4.76	0.40	1.14
hGH:				
183	Auramine O	0.72	4.00	0.70
240	Carminic acid	0.63	5.26	0.80
443	Sulfamethazine	0.60	4.76	0.79
512	Amaranth	0.81	5.26	0.68

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INHIBITION GCSF	hGH	MMTV
541	5-Bromo-4-Chloro-3-indoxyl-phosphate K-salt	0.90	6.25	0.86
556	Chromazurol S	0.73	33.33	0.87
561	Clove Oil	0.62	5.00	0.05
577	Na-Ne-Diacetyl-L-lysine	0.64	4.00	0.68
578	Dibenzoyl-D-tartaric acid	0.65	4.00	0.91
630	Hydantoin-5-acetic acid	0.70	3.57	0.74
640	Kernechtrot	0.64	5.00	0.59
759	Piperidine	0.64	5.88	0.95
764	Prednisolone	0.82	4.54	0.59
875	Black Walnut extract	0.69	6.25	0.80
892	Colts Foot Leaves extract	0.68	11.11	0.87
893	Comfrey Leaf extract	0.74	11.11	0.90
920	Horehound Herb extract	0.56	3.84	0.84
921	Horsetail Grass extract	0.72	3.44	0.86
942	Pau D'Arco extract	0.80	6.25	0.63
970	Thyme extract	0.57	4.34	1.07
1591	1,2-Bis(di-p-tolylphosphino)-ethane	0.56	5.55	0.96
1604	2,4-Bis[5,6-bis(4-sulfophenyl)-1,2,4-Triazine-3-yl)-pyridine, tetrasodium salt hydrate	0.77	5.00	0.97
1635	[(15)-endo]-(-)-Borneol	0.71	9.09	0.99
1640	1,2-Bis(2-pyridyl)-ethylene	0.79	5.00	0.59
1641	2,3-Bis(2-pyridyl)-pyrazine	0.83	5.55	0.60

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INHIBITION GCSF	hGH	MMTV
1648	2-[5,6-Bis(4-sulfophenyl)-1,2,4-triazine-3-yl]-4-(4-sulfophenyl)-pyridine, trisodium salt	0.86	7.69	1.00
1651	Bis(2,2,2-trifluoroethyl) (methocarbonyl-methyl)-phosphonate	0.69	3.57	0.70
1655	2,5-Bis(trifluoro-methyl)benzoic acid	0.54	4.76	0.81
1703	3-Bromobenzonitrile	0.76	10.00	0.90
1704	4-Bromobenzonitrile	0.77	4.16	0.94
1705	4-Bromobenzophenone	0.54	14.28	0.62
1712	Calcein Blue	0.74	8.33	0.94
1720	(15)-(-)-Camphor	0.65	4.76	0.66
1764	7-(Carboxymethoxy)-4-Methylcoumarin	0.55	7.14	0.82
1770	Carminic acid	0.54	10.00	0.57
1771	L-Carnosine	0.71	10.00	0.72
1773	O-Cresolphthalein Complexone	0.62	10.00	0.67
1890	Alloxazine	0.80	5.26	0.58
2035	5-Bromofuroic acid	0.57	7.14	0.89
2036	8-Bromoguanosine	0.58	4.34	0.81
2037	1-Bromohexadecane	0.51	4.00	0.50
MMTV:				
2010	2-Bromo-4,6-dinitroaniline	0.80	0.63	3.57

To determine the number of lead chemicals, which reproducibly score as positives in repeated luciferase assays, two types of experiments were conducted:

1) G-CSF lead chemicals #1780, #58, #1783, #1374 were subjected to 48 independent luciferase assays performed on the same day. Compounds #58, #1780 and #1374 scored as positives in every single one of these assays inducing luciferase expression between 2 and 28 fold (#58), 20 and 80 fold (#1780) and 5 and 40 fold (#1374). Probably due to its relatively low induction of luciferase expression (1.5 to 8 fold), Compound #1783 scored as positive only in half of the 48 repeat assays.

2) All of the 18 lead chemicals inducing luciferase expression from the MMTV promoter were again subjected to luciferase assays: 10 chemicals (#453, #519, #562, #765, #828, #848, #1269, #1316, #1384 and #2148) again induced luciferase expression between 2.1 and 2.8 fold. Probably due to the relatively low induction level close to the background of the assay, the other eight lead chemicals did not repeat on that particular day. The most prominent lead chemical, #453 (13.3 fold induction in the original high-throughput assay), was repeated in a total of 3 independent assays and consistently induced luciferase expression from the MMTV promoter between 10 and 35 fold. Replacing DMSO by methanol to dissolve the chemical did not affect its ability to activate the MMTV promoter.

2. Screen II

In another high throughput screen (screen II), aqueous clarified supernatants derived from individual Actinomyces colonies prepared by standard methods, as well as

corresponding methanol extracts were subjected at 1:10 initial dilution to a fully automated, robotic High-Throughput luciferase assay using the system described in U.S. patent application #382,483. Out of 356 samples tested for modulation of the G-CSF (clone G1002), GM-CSF (clone GM1105) and MMTV (clone M10) promoters, 25 samples scored as positives, 7 of which were promoter-specific. A summary of the obtained results is contained in Figures 56 and 57.

3. Screen III

In yet another independent high throughput screen (screen III), 500 compounds, consisting of 96 fermentation broths and 404 pure chemicals, were tested against a G-CSF (G1002) reporter cell line and an MMTV reporter control cell line. The number of lead compounds identified in this screen are shown in table 2.

Thus high-throughput screening of fermentation broth samples using a luciferase expression assay can consistently lead to the discovery of samples with the potential to be developed into novel pharmaceuticals.

D. Effects of Lead Chemicals on Endogenous G-CSF mRNA Levels

Northern blot analysis was used to demonstrate the stimulatory effects of lead chemicals #670 and #1255 on endogenous G-CSF mRNA levels. As shown in Figure 58, both OSI #670 and #1255 stimulated production of G-CSF mRNA, as shown by a G-CSF-specific probe, but not of actin mRNA, as shown by a β-actin-specific probe. Also shown are the effects of the solvent, DMSO, used to dissolve the chemicals and a proteinaceous positive regulator, interferon-gamma. From these data it is concluded that

chemicals, which induce luciferase expression from specific promoters, in plasmids stably integrated into cells, are also capable of stimulating mRNA production from the corresponding endogenous promoters.

To further confirm, that compounds that had been identified in a luciferase expression assay using a G-CSF specific reporter cell line would be active in inducing transcription of the endogenous G-CSF gene, cells from the parental cell line 5637 used to construct the reporter cell line were incubated with cycloheximide (25 μg/ml), DMSO (0.5%,solvent control) and low, medium and high concentrations of the compounds 542 (10, 50, 250 μM),1255 (20, 100, 500 μM), 1793 (0.25, 1.2, 6.25 μM) and 1904 (20, 100 μM) for 18 hours. RNA was extracted and the concentration of G-CSF, GM-CSF and gamma-actin mRNA was determined by the S1 protection method as described in Materials and Methods. The positions of G-CSF GM-CSF and gamma-actin specific protected fragments are indicated (G, GM, A) at the left side of the gel (Figure 59).

Interestingly, all four compounds tested increased the amount of G-CSF mRNA and at least two of them, namely #542 and #1793, also increased the amount of GM-CSF mRNA. Compound #543, a structural analog of #542 showed similar activity .

Table 3

SUMMARY OF HIGH THROUGHPUT SCREEN III

Number (%) of Chemicals Which Activate Expression:

	2-3X	3-5X	5-7X	7-10X	>10X	Total
G-CSF	0 (0%)	2 (0.4%)	0 (0%)	0 (0%)	0 (0%)	2 (0.4%)
MMTV	2 (0.4%)	2 (0.4%)	3 (0.6%)	1 (0.2%)	1 (0.2%)	9 (1.8%)

CYTOTOXIC COMPOUNDS: 5 (1%)

E. Dose response Analysis of Structurally Related Lead Chemicals

Among the chemicals which specifically activated the G-CSF promoter were groups of structural homologs. Three such homologs, #80, #670, and #1780, belong to groups listed in Table 2. These three structurally related chemicals all specifically activated the G-CSF promoter. Dose response graphs obtained with chemicals #80, #670, and #1780 are shown in Figure 60. Although these chemicals all demonstrate large maximal stimulations, it is clear that their potencies, as measured by their ED50's (concentration of chemical resulting in 50%

maximal stimulation), show wide variability (5-70 μM)).

F. Effects of Lead Chemicals on Target Protein Secretion

Lead chemicals (# 542 and #1780), which were shown to stimulate levels of endogenous G-CSF mRNA as well as luciferase expression from the G-CSF promoter/luciferase fusion constructs, were further investigated for their ability to increase G-CSF secretion into the media of 5637 bladder carcinoma cells incubated with the chemicals for 48 hours. The levels of G-CSF in the cell supernatants were determined by a sandwich-antibody assay as described in Materials and Methods (Figure 61).

G. Cytotoxicity of Lead Chemical #542

To address the question whether the induction of G-CSF, and GM-CSF transcription by the compound #542 was a specific effect or rather a phenomenon linked to a potential sensitivity of these promoters to stress exerted by toxic compounds, the concentration dependency of induction of luciferase activity in the reporter cell line G21 was compared to the concentration dependency of inhibition of respiration in FRE cells.

Cells were seeded into 96 well microtiter plates (20,000 cells/well) and cultured overnight. Compound #542 was added at various concentrations and the cells were incubated for 6 hours. Luciferase activity was determined as described in Materials and Methods. The MTT-colorimetric assay was carried out on identically treated samples of FRE cells. Induction of luciferase reporter signal (plain line) and on inhibition of respiration (dashed line) are plotted versus the concentrations of compound (Figure 62). The ED50 for

induction of luciferase activity differed from the ED50 for inhibition of respiration by a factor of almost 10, which might indicate that compound #542 exerts a specific effect on G- and GM-CSF transcription.

5

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(B) COMPUTER: IBM PC compatible
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCGACCCGGG CGGCCGCTGA TCAGACGTCG GGCCCGGTAC CGTGCACTAC
GTAAGATCTA 60

AGCTT
65

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACTAGTCTGC AGGCTAGCAC TCTTCTGGTC CCCACAGACT CAGAGAGAAC
CCACCATGGA 60

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACGCCAAAA ACATCAAGAA AGGCCCGGCG CCATTCTATC CTCTAGAGGG
GATCCAGCTG 60

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAGATCTTAC GTAGTGCACG GTACCGGGCC CGACGTCTGA TCAGCGGCCG
CCCGGG 56

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGGGTTCT CTCTGAGTCT GTGGGGACCA GAAGAGTGCT AGCCTGCGAC
TAGTAAGCT 59

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATTCAGCTG GATCCCCTCT AGAGGATAGA ATGGCGCCGG GCCTTTCTTG
ATGTTTTTGG 60

CGTCTTCCAT
70

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCGGCCCC TAGGGCCGCG GCCGCAT
27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGCGGCCGC GGCCCTAGGG GCC
23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCGGCCCT AGGGGCGGCC GCAT
24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGCGGCCGC GGCCCCCTAG GGCC
24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTGGCCC CTAGGGCCAC TAGTCTGCAG CTATGATGAC ACAAACCCCG
CCCAGCGTCT 60

TGTCATTGGC GA
72

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCGGGGATC CCGGTGATCA GACTCGATAC TACTGTGTTT GGGGCGGGTC
GCAGAACAGT 60

AACCGCTTAA GCT
73

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATTCGAACAC GCAGATGCAG TCGGGGCGGC GCGGTCCGAG GTCCACTTCG
CATATTAAGG 60

TGACGCGTGT GGG
73

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGTGCGTCTA CGTCAGCCCC GCCGCGCCAG GCTCCAGGTG AAGCGTATAA
TTCCACTGCG 60

CACACCCGAT C
71

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCGGCCCC TAGGGCCATT T
21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCGGGGATCC CGGTAAA
17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTTTTTGTT CCAACCCCCC TGCATT
26

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCCTGCATTG TCTTGGACAC CAAAT
25

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCGCTCCAGG AGAAGCTGGT GAGT
24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGCTGATGG GTGAGTGTCT TGGC
24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATCAGCGGCT CAGCCTTCTT

20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGTGACCACA AAATGCCAGG GAGGCGGG
28

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCAGGCCACA GTGCCCAAGA GACAGCAGCA GGCT
34

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AATTCGGTCA CCATTAATCA TTTCCTCTGT GTATTTAAGA GCTCTTTTGC
CAGTGAGCCC 60

AGTACACAG
69

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCCAGTGGTA ATTAGTAAAG GAGACACATA AATTCTCGAG AAAACGGTCA
CTCGGGTCAT 60

GTGTCTCTCT TTCCG
75

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGAGAAAGGC TAAAGTTCTC TGGAGGATGG AAGACGCCAA AAACATCAAG
AAAGGCCCGG 60

CGCCATTCTA TCCT
74

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATTTCAAGAG ACCTCCTACC TTCTGCGGTT TTTGTAGTTC TTTCCGGGCC GCGGTAAGAT 60

AGGAGATC 68

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCGGCGCGGT CATACGGGCA GCTGG 25

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGCCCGTAT GGA 13

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATGAGAATA TCACTGTCCC AGACACCAAA GTTAATTTCT ATGCCTGGAA
50

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTCCAGGCAT AGAAATTAAC
20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCCGGTGTGG TCACCCGGCG CGCCCCAGGT CGCTGAGGGA CCCCGGCCAG
GCGCGGA 57
(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CATCTCCGCG CCTGGCCGGG GTCCCTCAGC GACCTGGGGC GCGCCGGGTG ACCACACCGG 60

GGGGCC 66

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GATGGAAGAC GCCAAAAACA TCAAGAAAGG CCCGGCGCCA TTCTATCCT 49

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CTAGAGGATA GAATGGCGCC GGGCCTTTCT TGATGTTTTT GGCGTCTTC 49

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ACCGCCGAGC TTCCCGGGAT CCGGGCCCCC GGTGTGGTCA
40

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTCCTGCCTG GCTGTGGCTT ATGGAAGACG CCAAAAACAT
40

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CAGTCCGAGC TCCATGGGGT CCAAGTTTTG
30

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CAGTAAGAGC TCAGCCCTTG CCCTGGGCAG G
31

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TAAGTGTGTT ATAATTTCAT CGATCATGTT
30

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCGGGGTTGT GGGCACCTTG CTGCTGCACA TATAAGGCGG GAGGTTGTTG
CCAACTCTTC 60

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AGAGCCCCAC GAAGGACCAG AACAAGACAG AGTGCCTCCT GCCGATCCAA ACATGGA 57

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ATTTCAAGAG ACCTCCTACC TTCTGCGGTT TTTGTAGTTC TTTCCGGGCC GCGGTAAGAT 60

AGGAGATC 68

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GTTTGGATCG GCAGGAGGCA CTCTGTCTTG TTCTGGTCCT TCGTGGGGCT CTGAAG 56

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CGCTGCGCTC GGGCTACCCA ATGCGTGGAC
30

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

AACAGCTAAA CGGAGTCGCC ACACCACTGT
30

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCGCTGCCTT TCCTTATGAA GAAGACACAA
30

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCAGAACAGC TAAACGGAGT CGCCACACCA CTGTTTGTGC
40

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

AAACAGTGGT GTGGCGACTC CGTTTAGCTG TTCTGGAGCT
40

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TGGATCGCAG CGCTGCCTTT CCT
23

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CATGAGGAAA GGCAGCGCTG CGATCCAGCA C
31

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TGGCGCAGCG CTCCAGGAGA AGCTG
25

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CGCTATGGAG TTGGCTCAAG CAGCCTGC
28

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

GGCGGGTCTG TAGGCAGGTC GGCTC
25

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CAGTAAGAGC TCAGCCCTTG CCCTGGGCAG G
31

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CTCCAGCCCG CAGCTCCAGG AGTCTG
26

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CCCTCTACAC TGGCAGTTCC ACCTG

25

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GGCCAAGGAG GCCGAGAATA TCACG
25

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

GCCAGACTTC TACGGCCTGC TGCCCGAC
28

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TCAGCAATTG AGAGCATTCT TAAA
24

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GTCCTTGATA TGGATTGGAT GTCG
24

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

ACTAATAATG TAAAAGACGT CACTAAATTG
30

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TCTCGCTTAT CCAACAATGA CTTGG
25

What is claimed is:

1. A method of directly transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the hematopoietic growth factor encoded by the gene which is expressed by the cell, which molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene encoding the hematopoietic growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

2. A method of claim 1, wherein the molecule does not naturally occur in any cell of a lower eucaryotic organism.

3. A method of claim 1, wherein the molecule does not naturally occur in any cell.

4. A method of claim 1, wherein the molecule is not a naturally occurring molecule.

5. A method of claim 1, wherein the cell is a cell of the multicellular organism.

6. A method of claim 1, wherein the cell is an animal cell.

7. A method of claim 6, wherein the animal cell is a human cell.

8. A method claim 1, wherein the transcriptional modulation comprises upregulation of expression of the gene encoding the hematopoietic growth factor.

9. A method of claim 1, wherein the transcriptional modulation comprises downregulation of expression of the gene encoding the hematopoietic growth factor.

10. A method of claim 1, wherein the molecule binds to a modulatable transcription sequence of the gene.

11. A method of claim 1, wherein the molecule comprises an antisense nucleic acid.

12. A method of claim 1 wherein the molecule comprises double-stranded nucleic acid.

13. A method of claims 1 wherein the molecule comprises a nucleic acid capable of forming a triple helix with double-stranded DNA.

14. A method of claim 1, wherein the hematopoietic growth factor is a colony stimulating factor.

15. A method of claim 14, wherein the colony stimulating factor is granulocyte-macrophage colony stimulating factor.

16. A method of claim 14, wherein the colony stimulating

factor is granulocyte colony stimulating factor.

17. A method of claim 14, wherein the colony stimulating factor is macrophage colony stimulating factor.

18. A method of claim 1, wherein the hematopoietic growth factor is erythropoietin.

19. A method of claim 1, wherein the hematopoietic growth factor is IL-3.

20. A method of claim 1, wherein the hematopoietic growth factor is stem cell factor.

21. A method of claim 1, wherein the hematopoietic growth factor is an interleukin.

22. A method of claim 1, wherein the hematopoietic growth factor is a cytokine.

23. A method of claim 1, wherein the hematopoietic growth factor is a lymphokine.

24. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the gene encoding the hematopoietic

growth factor, and (iii) a DNA sequence encoding a polypeptide other than the hematopoietic growth factor, which polypeptide is capable of producing a detectable signal, and which DNA sequence is coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable detectable signal to be produced by the polypeptide so expressed, quantitatively determining the amount of the signal produced, comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

25. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the gene encoding the hematopoietic growth factor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the

control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable change in the amount of the polypeptide produced, quantitatively determining the amount of the polypeptide so produced, comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the amount of the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

26. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a hematopoietic growth factor, which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the hematopoietic growth factor, (ii) a promoter of the gene encoding the hematopoietic growth factor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the hematopoietic growth factor, causes a measurable

difference in the amount of mRNA transcribed from the DNA sequence, quantitatively determining the amount of the mRNA produced, comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable mRNA amount of, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the hematopoietic growth factor.

27. A method of any of claims 24, 25 or 26, wherein the sample comprises cells in monolayers.

28. A method of any of claims 24, 25 or 26, wherein the sample comprises cells in suspension.

29. A method of any of claims 24, 25 or 26, wherein the cells comprise animal cells.

30. A method of claim 29, where the animal cells are human cells.

31. A method of any of claims 24, 25 or 26, wherein the predefined number of cells is from about 1 to about 5×10^5 cells.

32. A method of claim 31, wherein the predefined number of cells is from about 2×10^2 to about 5×10^4 cells.

33. A method of any of claims 24, 25 or 26, wherein the predetermined amount of the molecule to be tested is based upon the volume of the sample.

34. A method of any of claims 24, 25 or 26, wherein the predetermined amount is from about 1.0 pM to about 20 μM.

35. A method of any of claims 24, 25 or 26, wherein the predetermined amount is from about 10 nM to about 500 μM.

36. A method of any of claims 24, 25 or 26, wherein the contacting is effected from about 1 to about 24 hours.

37. A method of claim 36, wherein the contacting is effected from about 2 to about 12 hours.

38. A method of any of claims 24, 25 or 26, wherein the contacting is effected with more than one predetermined amount of the molecule to be tested.

39. A method of any of claims 24, 25 or 26, wherein the molecule to be tested is a purified molecule.

40. A method of any of claims 24, 25 or 26, wherein the modulatable transcriptional regulatory sequence comprises a cloned genomic regulatory sequence.

41. A method of any of claims 24, 25 or 26, wherein the DNA consists essentially of more than one modulatable transcriptional regulatory sequence.

42. A method of either claim 24 or 25, wherein the DNA sequence encoding the polypeptide is inserted downstream of the promoter of the gene encoding a hematopoietic growth factor by homologous recombination.

43. A method of claim 24, wherein the polypeptide is a luciferase.

44. A method of claim 24, wherein the polypeptide is chloramphenicol acetyltransferase.

45. A method of claim 24, wherein the polypeptide is β glucuronidase.

46. A method of claim 24, wherein the polypeptide is β galactosidase.

47. A method of claim 24, wherein the polypeptide is neomycin phosphotransferase.

48. A method of claim 24, wherein the polypeptide is guanine xanthine phosphoribosyltransferase.

49. A method of claim 24, wherein the polypeptide is alkaline phosphatase.

50. A method of claim 25, wherein the polypeptide is capable of complexing with an antibody.

51. A method of claim 25, wherein the polypeptide is capable of complexing with biotin.

52. A method of claim 26, wherein mRNA is detected by quantitative polymerase chain reaction.

53. A screening method according to any of claims 24, 25 or 26 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a

predetermined amount of each different molecule to be tested.

54. A screening method of claim 53, wherein the plurality of samples comprises more that about 10^4 samples.

55. A screening method of claim 53, wherein the plurality of samples comprises more than about 5 X 10^4 samples.

56. A method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding hematopoietic growth factors which comprises essentially simultaneously screening the molecules against the genes encoding the hematopoietic growth factors according to the method of claim 53.

57. A screening method of any of claims 55 or 56, where more than about 10^3 samples per week are contacted with different molecules.

58. A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding a hematopoietic growth factor, the expression of which is associated with a defined physiological or pathological effect in the organism, which comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism and (b) specifically

transcriptionally modulates expression of the gene encoding a hematopoietic growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

59. A method of claim 58, wherein the molecule binds to a modulatable transcription sequence of the gene.

60. A method of claim 58, wherein the molecule comprises an antisense nucleic acid.

61. A method of claim 58, wherein the molecule comprises a double-stranded nucleic acid molecule.

62. A method of claim 58, wherein the molecule comprises a nucleic acid capable of forming a triple helix with double-stranded DNA.

63. A method of claim 58 wherein the multicellular organism is a human being.

64. A method of claim 58, wherein the multicellular organism is an animal.

65. A method of claim 63, wherein the physiological effect is the protection of a hematopoietic system from damage by chemotherapeutic agents.

66. A method of claim 63, wherein the physiological effect is the protection of stem cells from damage by chemotherapeutic agents.

67. A method of claim 63, wherein the defined pathological effect is a disorder and modulated expression of the gene encoding a hematopoietic growth factor is associated with amelioration of the disorder.

68. A method of claim 63, wherein the defined pathological effect is a hematopoietic dysfunction.

69. A method of claim 63, wherein the defined pathological effect is a tissue inflammation.

70. A method of claim 63, wherein the defined pathological effect is atherosclerosis.

71. A method of claim 63, wherein the defined pathological effect is a viral infection.

72. A method of claim 63, wherein the defined pathological effect is anemia.

73. A method of claim 63, wherein the defined pathological effect is leukopenia.

74. A method of claim 63, wherein the defined pathological effect is neutropenia.

75. A method of claim 63, wherein the defined pathological effect is cancer.

76. A method of claim 63, wherein the defined pathological effect is thrombocytopenia.

77. A method of claim 63, wherein the defined pathological effect is a dysfunction in a

cholesterol or other metabolic pathway.

78. A method of claim 63, wherein the administering comprises topical contact.

79. A method of claims 63, wherein the administering comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration.

Figure 1.
Features of the Mammalian Vector pUV102 with Inserted TK-NEO Cassette.

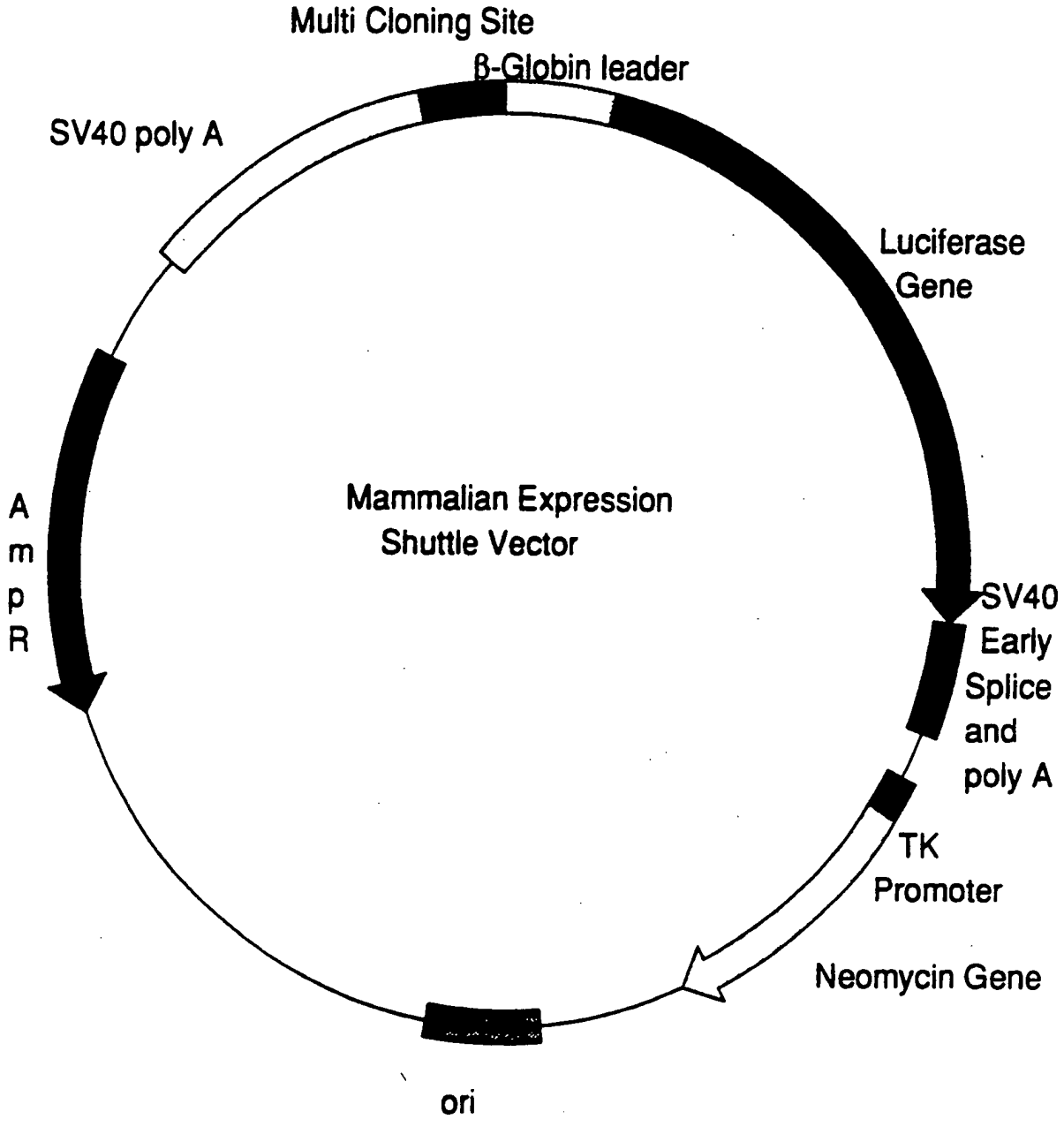


Figure 2.
pDO432.

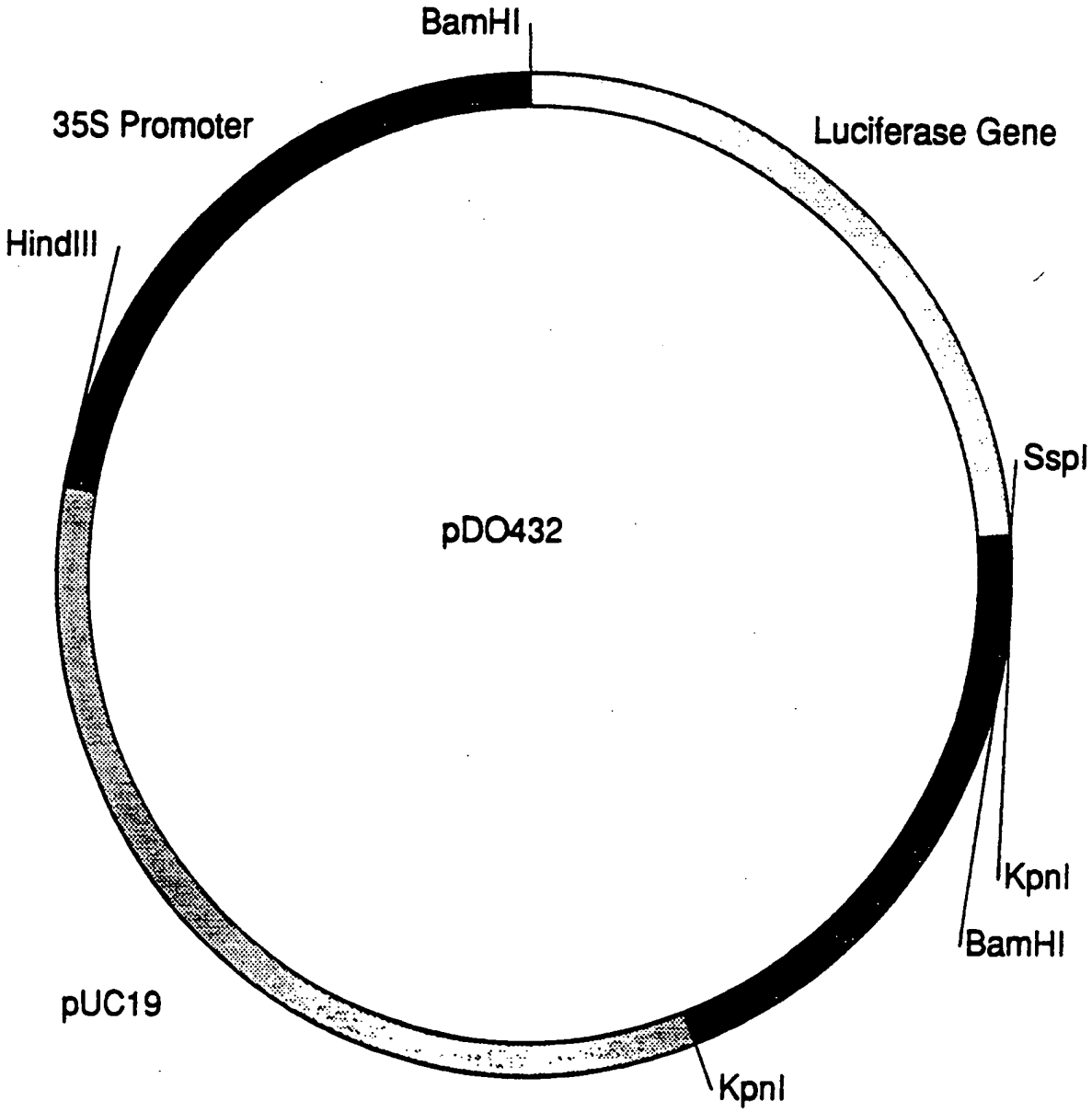


Figure 3.
pSVLuci.

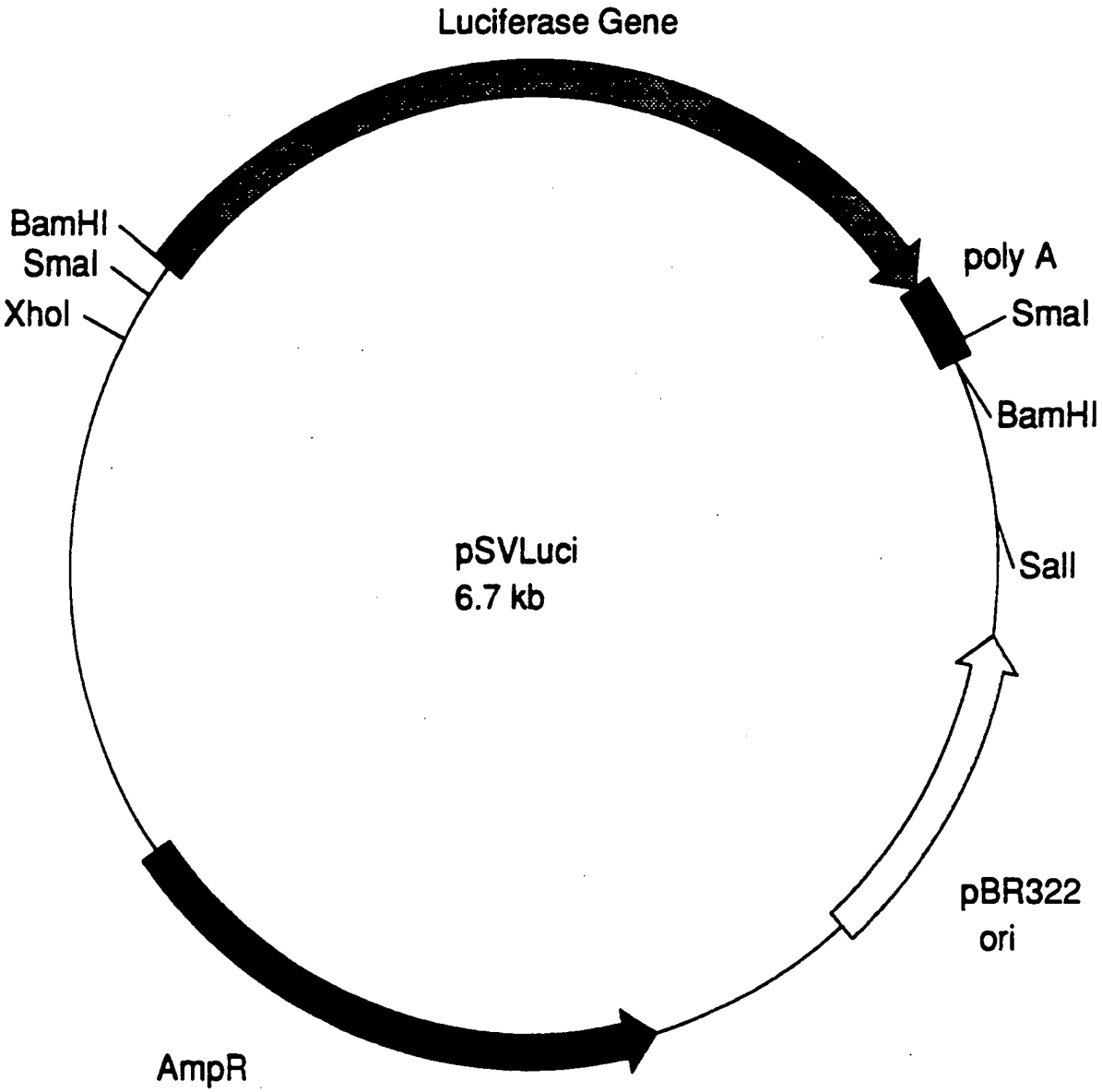


Figure 4.
pMLuci.

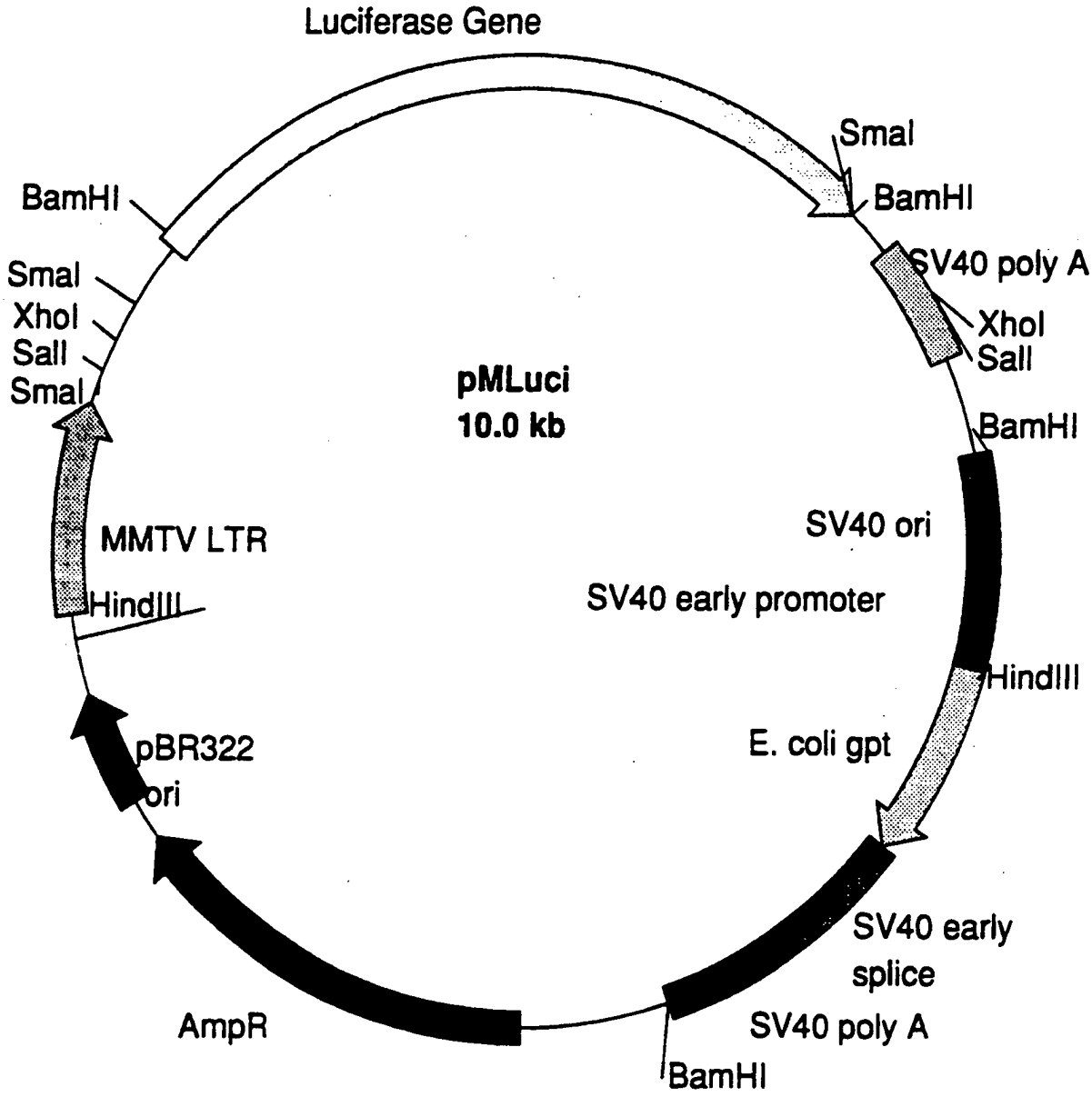


Figure 5.
Sequence of the pUV oligonucleotides.

pUV1:
5'TCGACCCGGGCGGCCGCTGATCAGACGTCGGGCCCGGTACCGTGCACTACGTAAGATCTAA
GCTT3'

pUV2:
5'ACTAGTCTGCAGGCTAGCACTCTTCTGGTCCCCACAGACTCAGAGAGAACCCACCATGGA
3'

pUV3:
5'AGACGCCAAAAACATCAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGGGATCCAGC
TG3'

pUV4:
5'TAGATCTTACGTAGTGCACGGTACCGGGCCCGACGTCTGATCAGCGGCCGCCCGGG3'

pUV5:
5'GGTGGGTTCTCTCTGAGTCTGTGGGGACCAGAAGAGTGCTAGCCTGCGACTAGTAAGCT3'

pUV6:
5'AATTCAGCTGGATCCCCTCTAGAGGATAGAATGGCGCCGGGCCTTTCTTGATGTTTTTGGCGT
CTTCCAT3'

Figure 6

Construction of pUV001

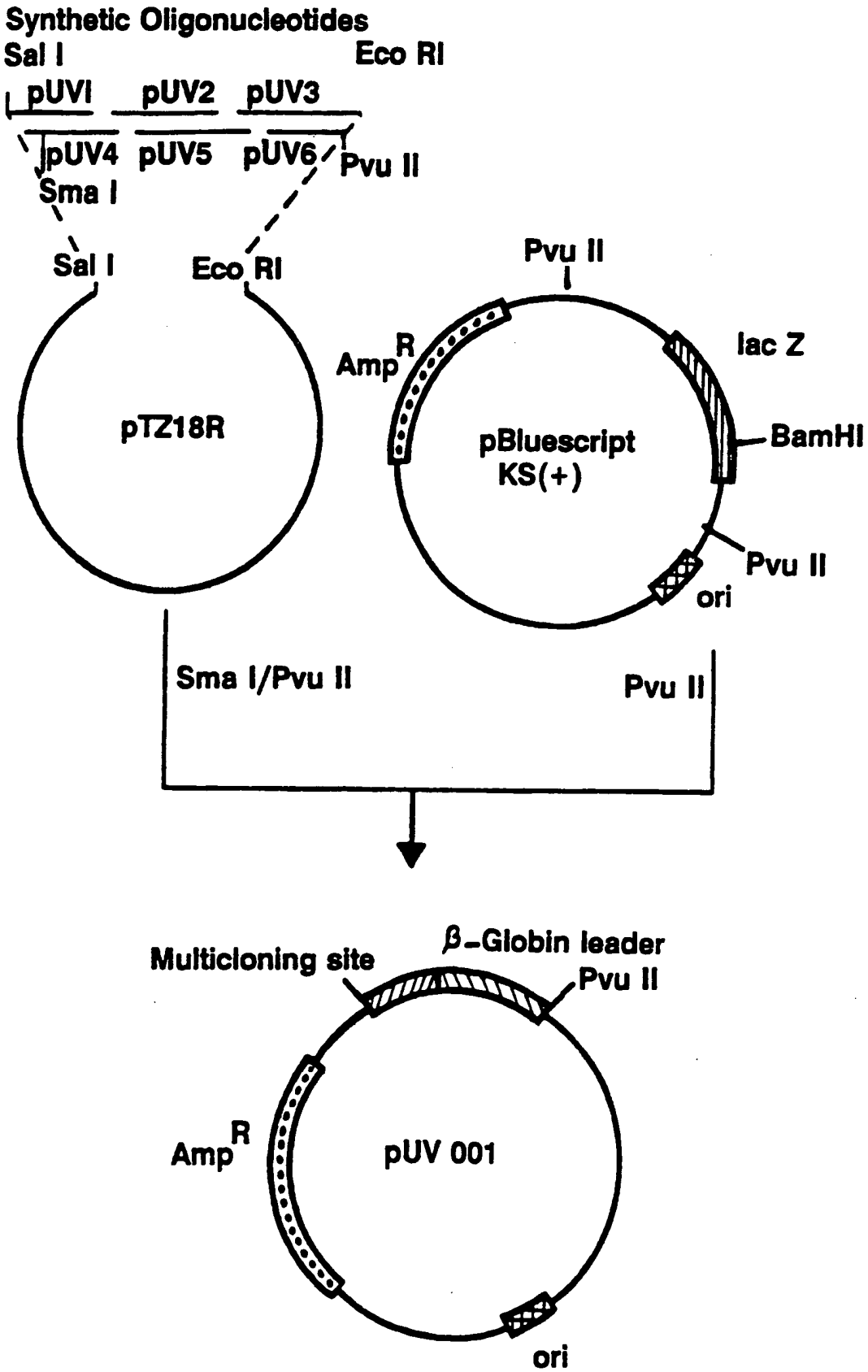
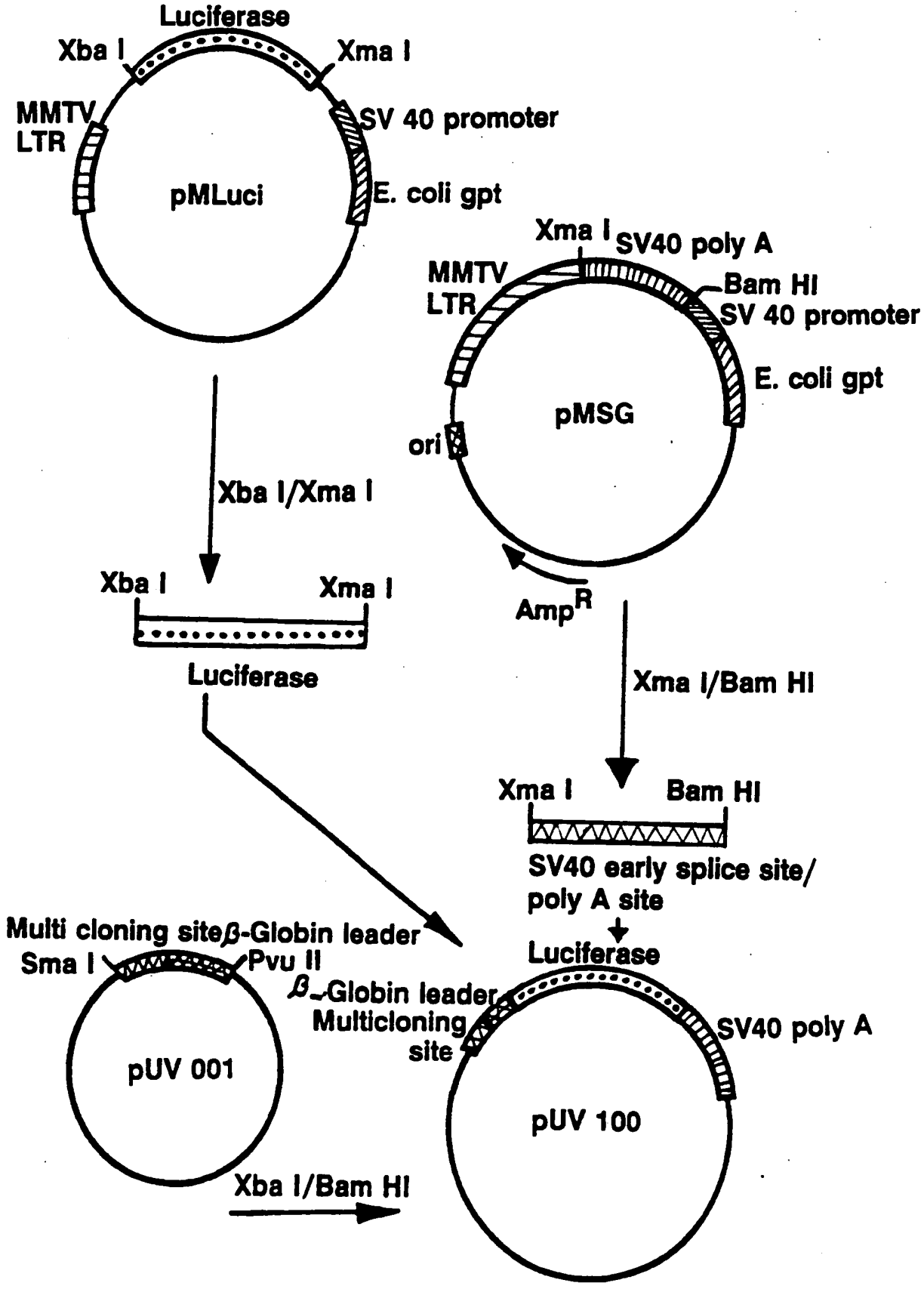


Figure 7

Construction of pUV100



8/63

Figure 8

Construction of pUV100-3

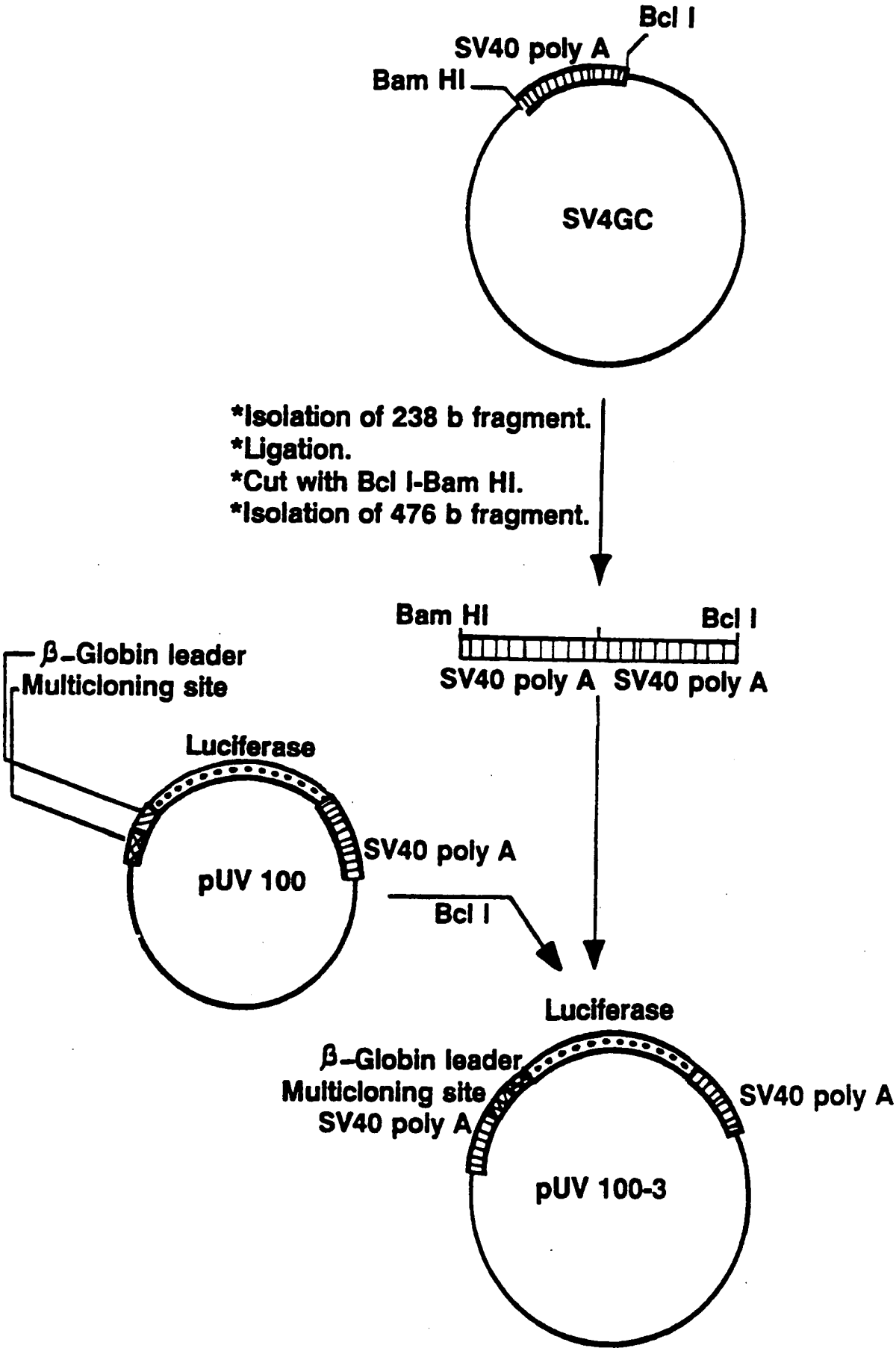


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Figure 9

Construction of pUV102 and pUV103

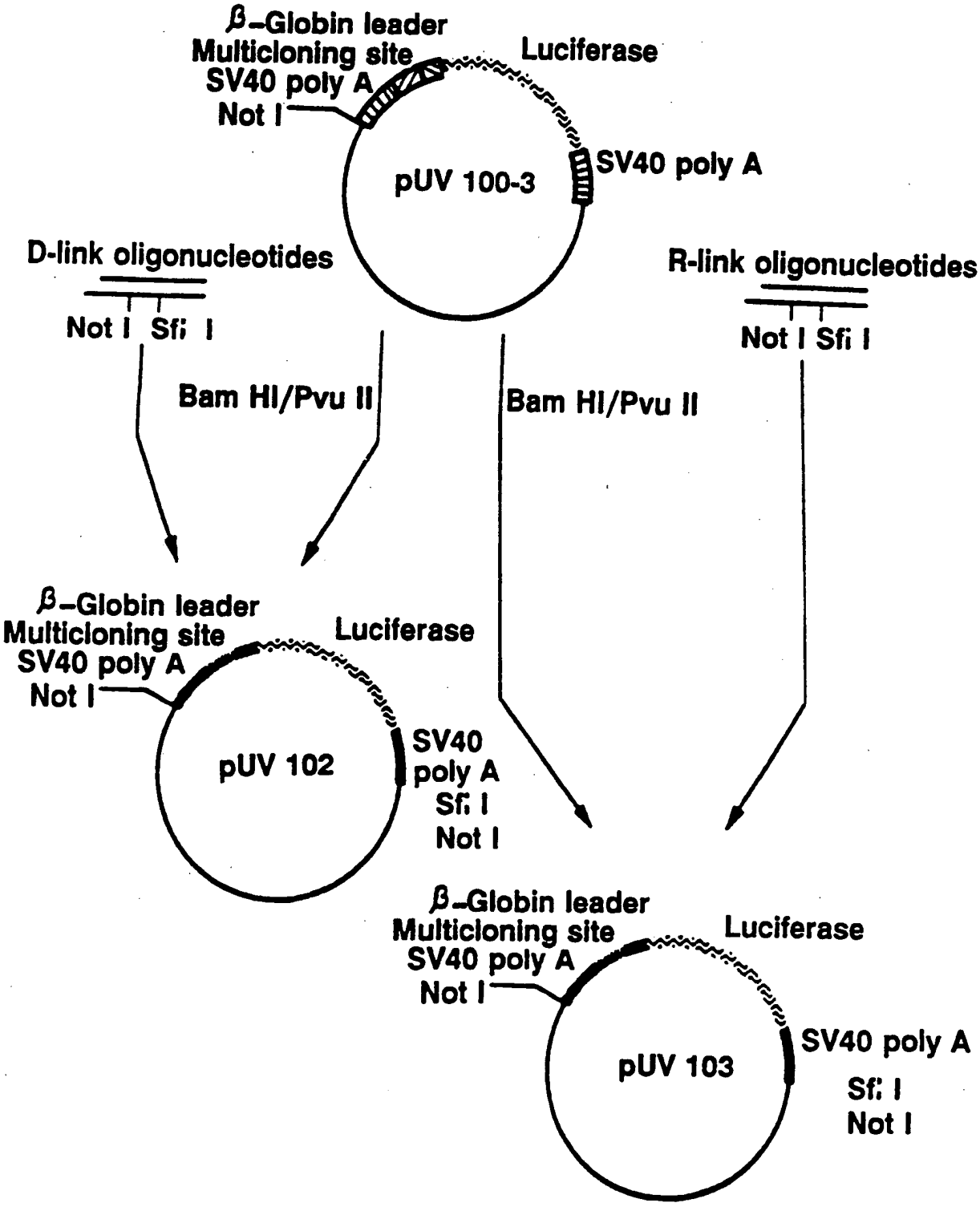


Figure 10

Synthetic HSV-TK Promoter

Oligo #1: 5' AGCTTGGCCCCTAGGGCCACTAGTCTGCAGCTATGATGACACAA ACCCCGCCCAGCGTCTTGTCATTGGCGA-3'

Oligo #2: 3' ACCGGGGATCCCGGTGATCAGACTCGATACTACTGTGTTTGGGG CGGGTCGCAGAACAGTAACCGCTTAAGCT-5'

Oligo #3: 5' ATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCGAGGTC CACTTCGCATATTAAGGTGACGCGTGTGGG-3'

Oligo #4: 3' TGTGCGTCTACGTCAGCCCCGCCGCGCCAGGCTCCAGGTGAAG CGTATAATTCCACTGCGCACACCCGATC-5'

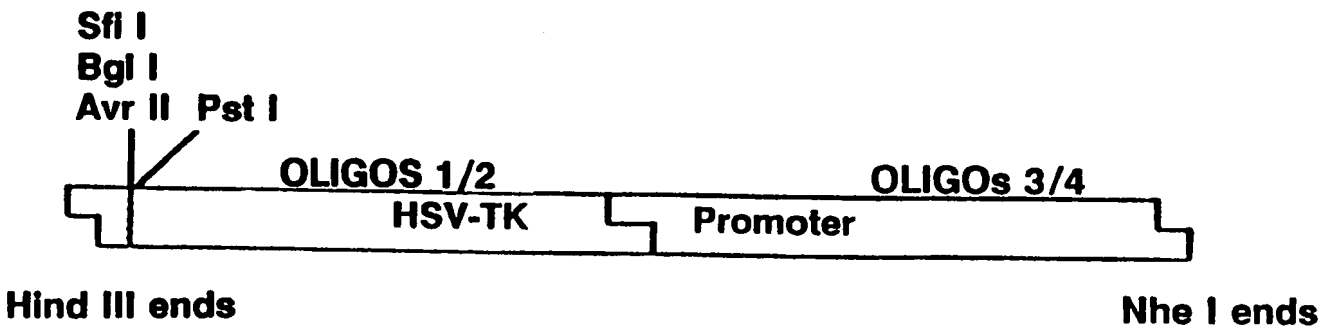


Figure 11

Constuction of pTKL100

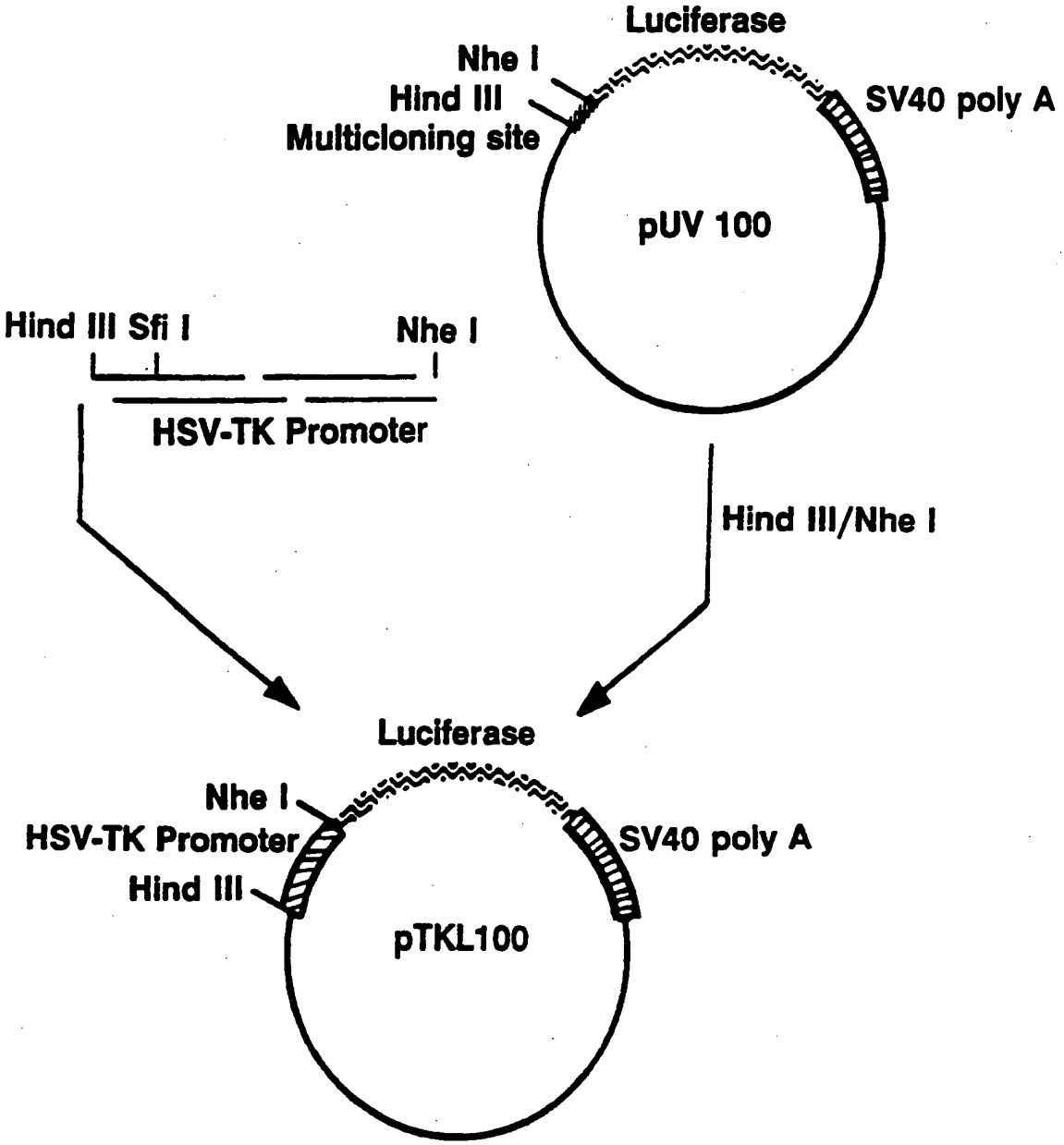


Figure 12

Construction of pTKNEO

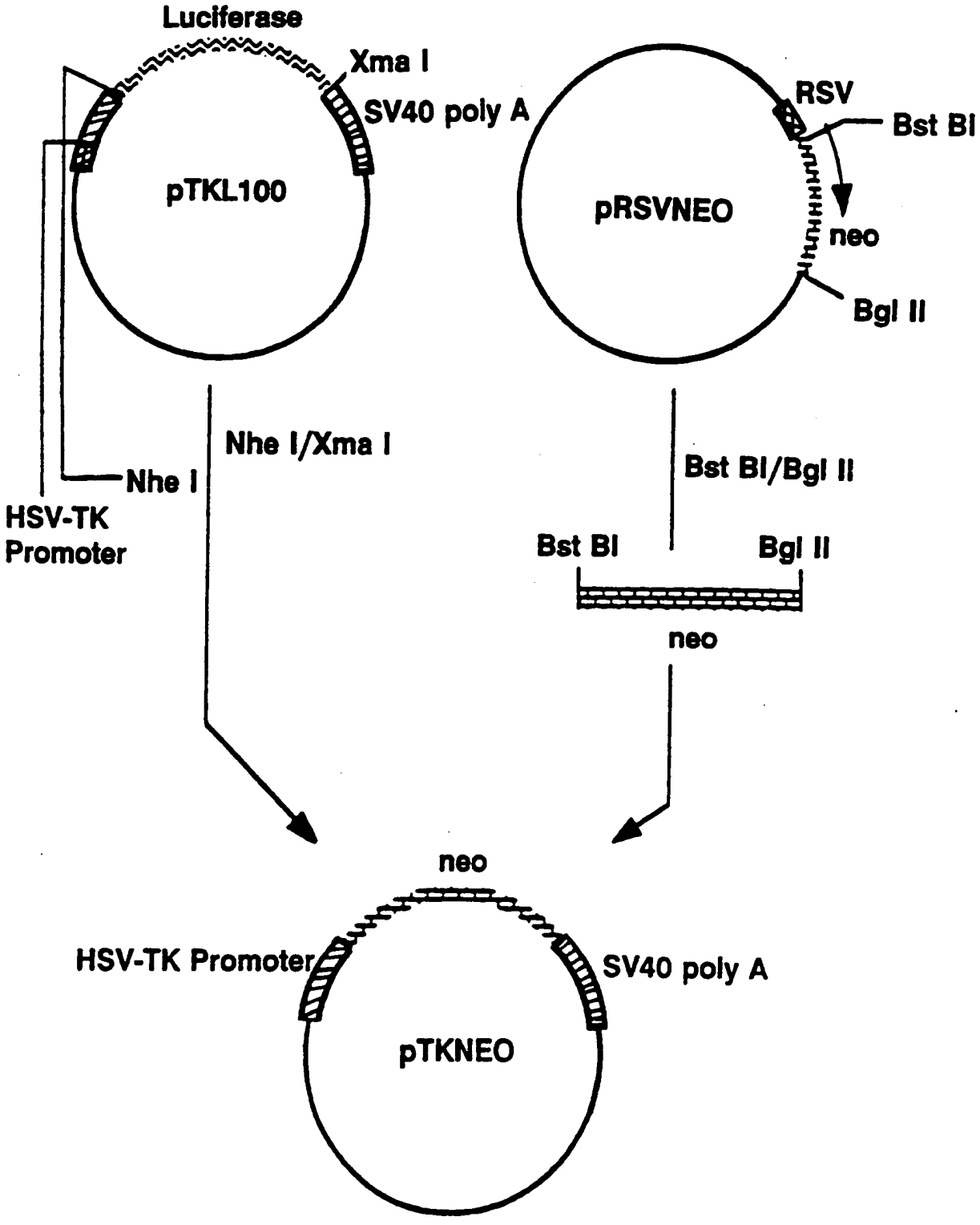


Figure 13

Construction of pTKNEO2

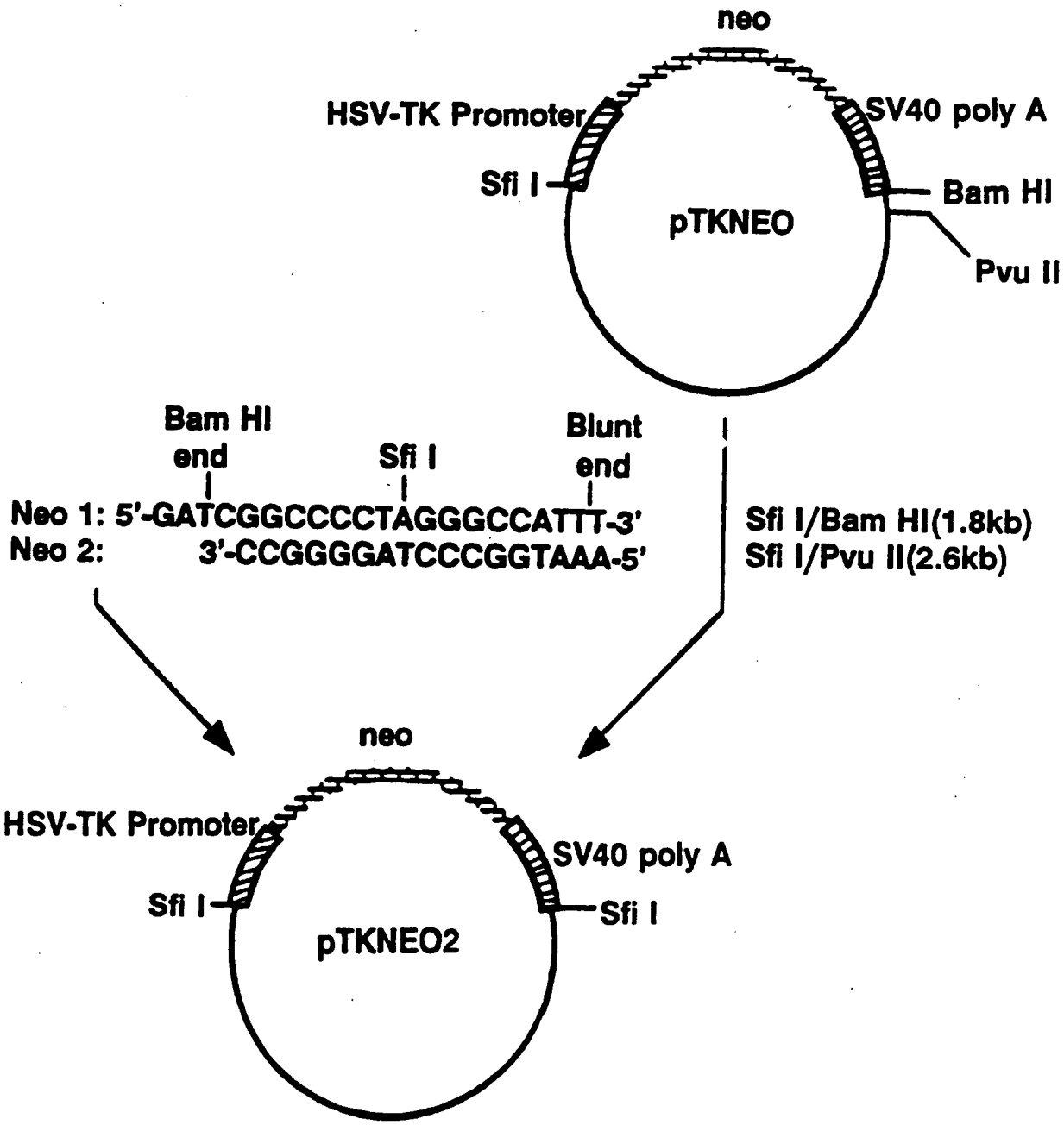


Figure 14

Construction of pTKNEO3

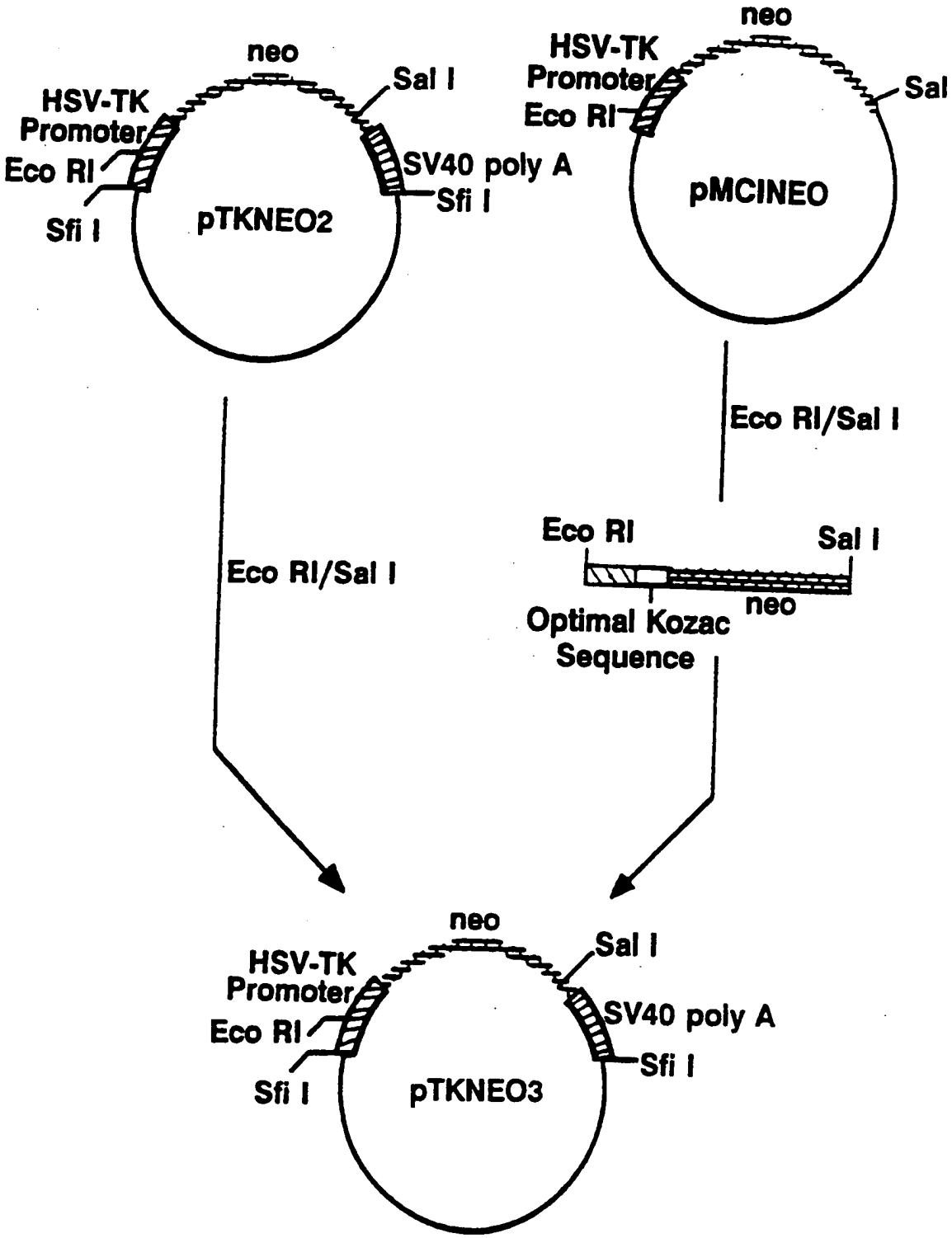


FIGURE 15. pJM710

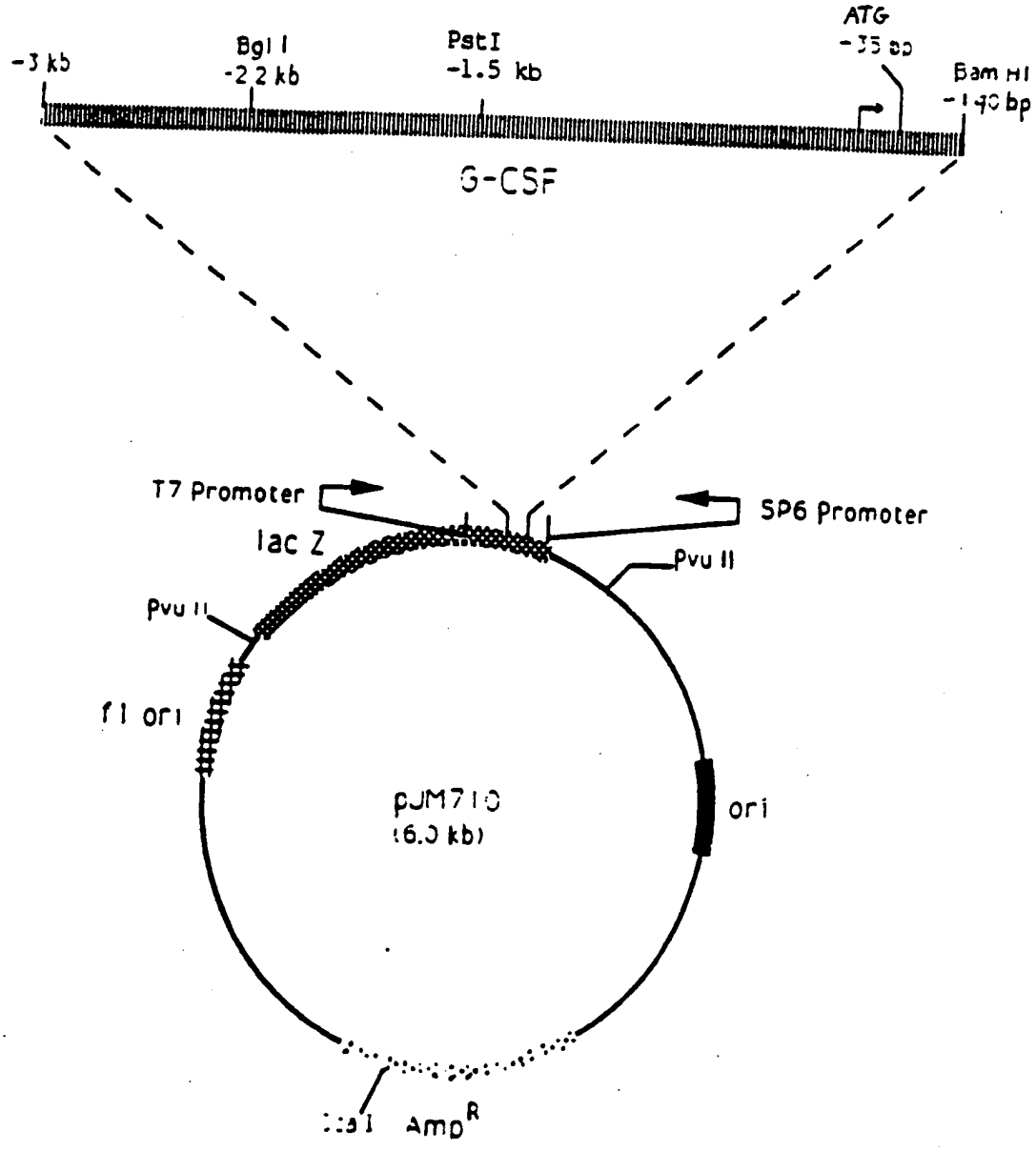


FIGURE 16. pGEM5 - Luci

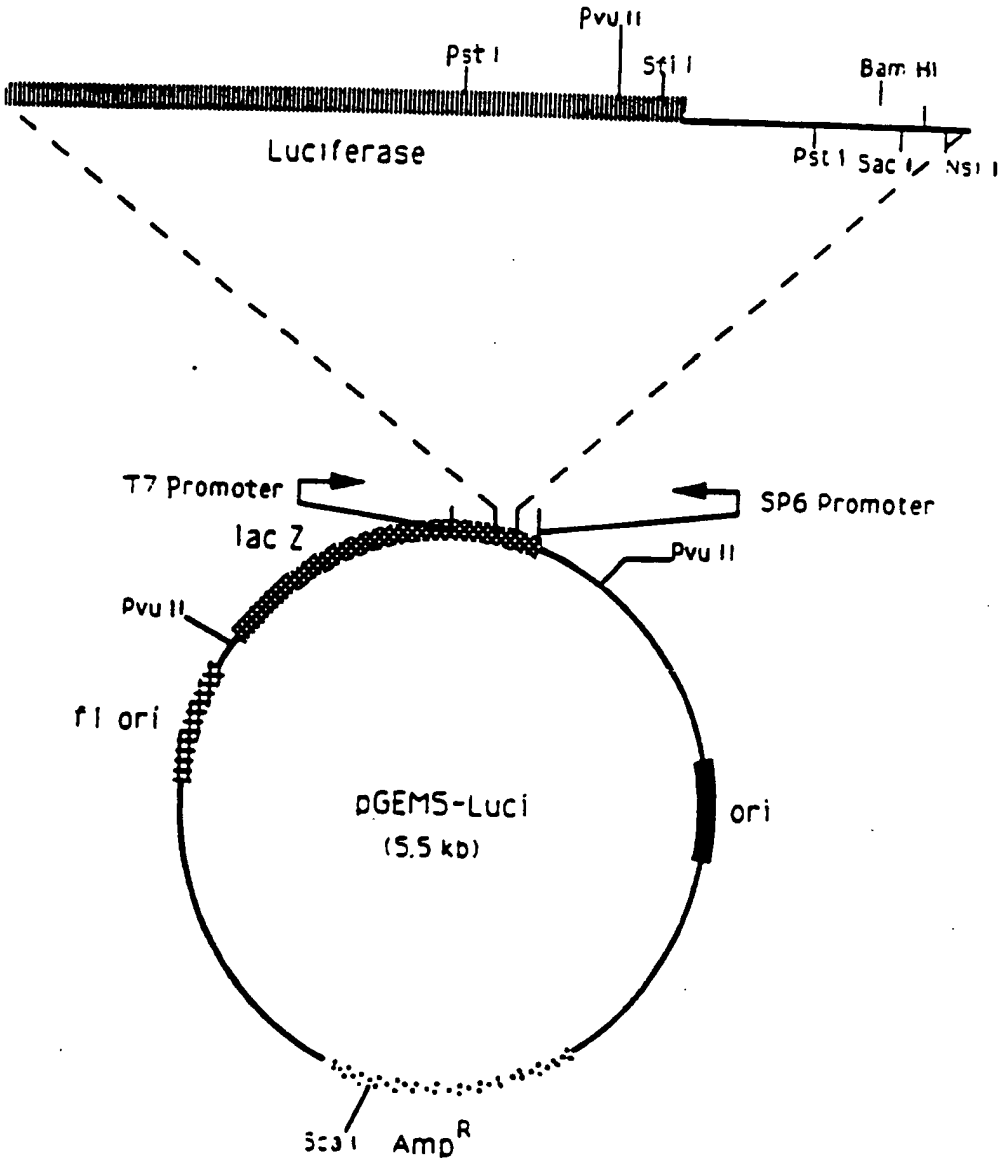


FIGURE 17. pG - Luc 1

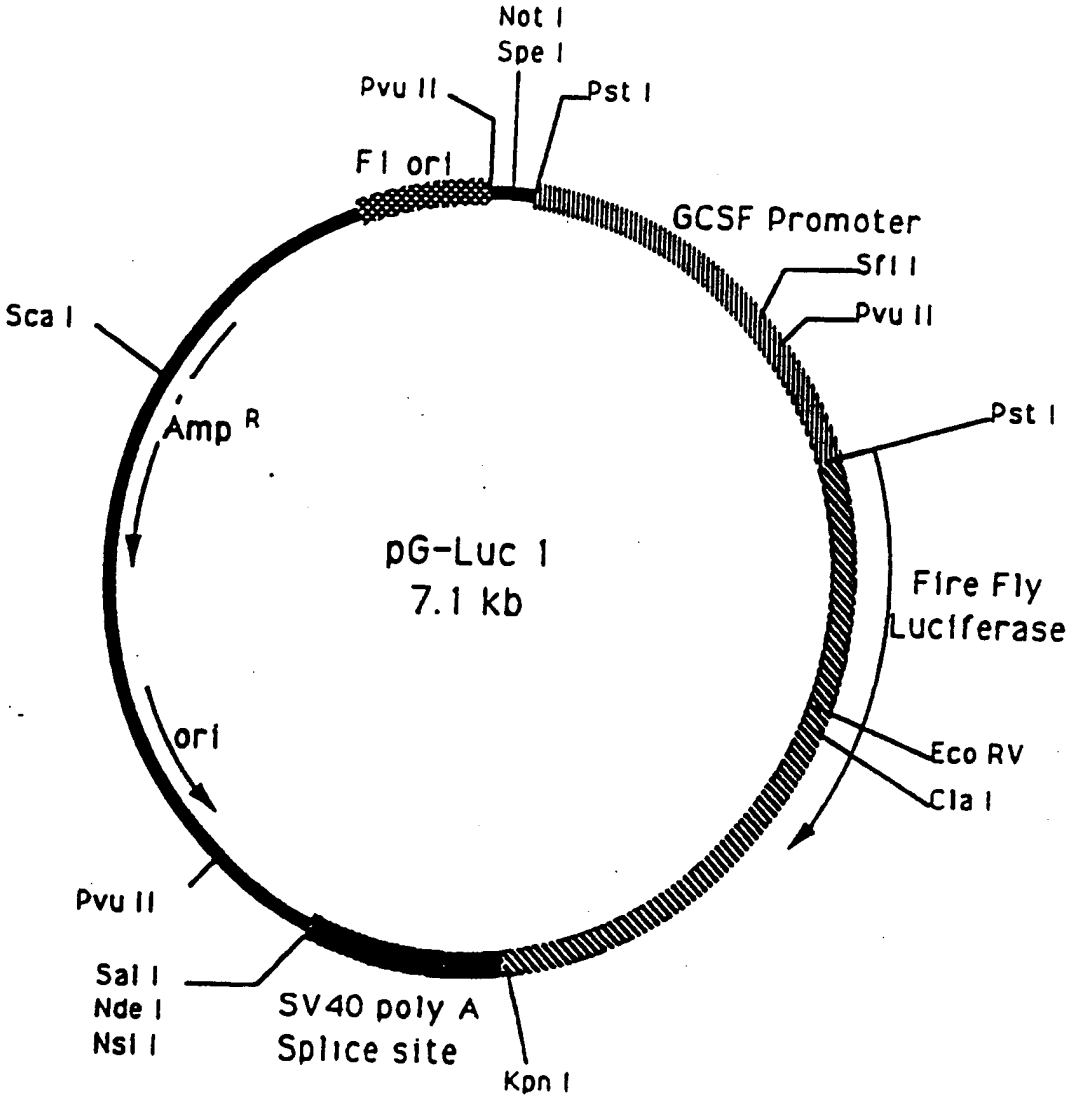


FIGURE 18. CONSTRUCTION OF pGUC 84

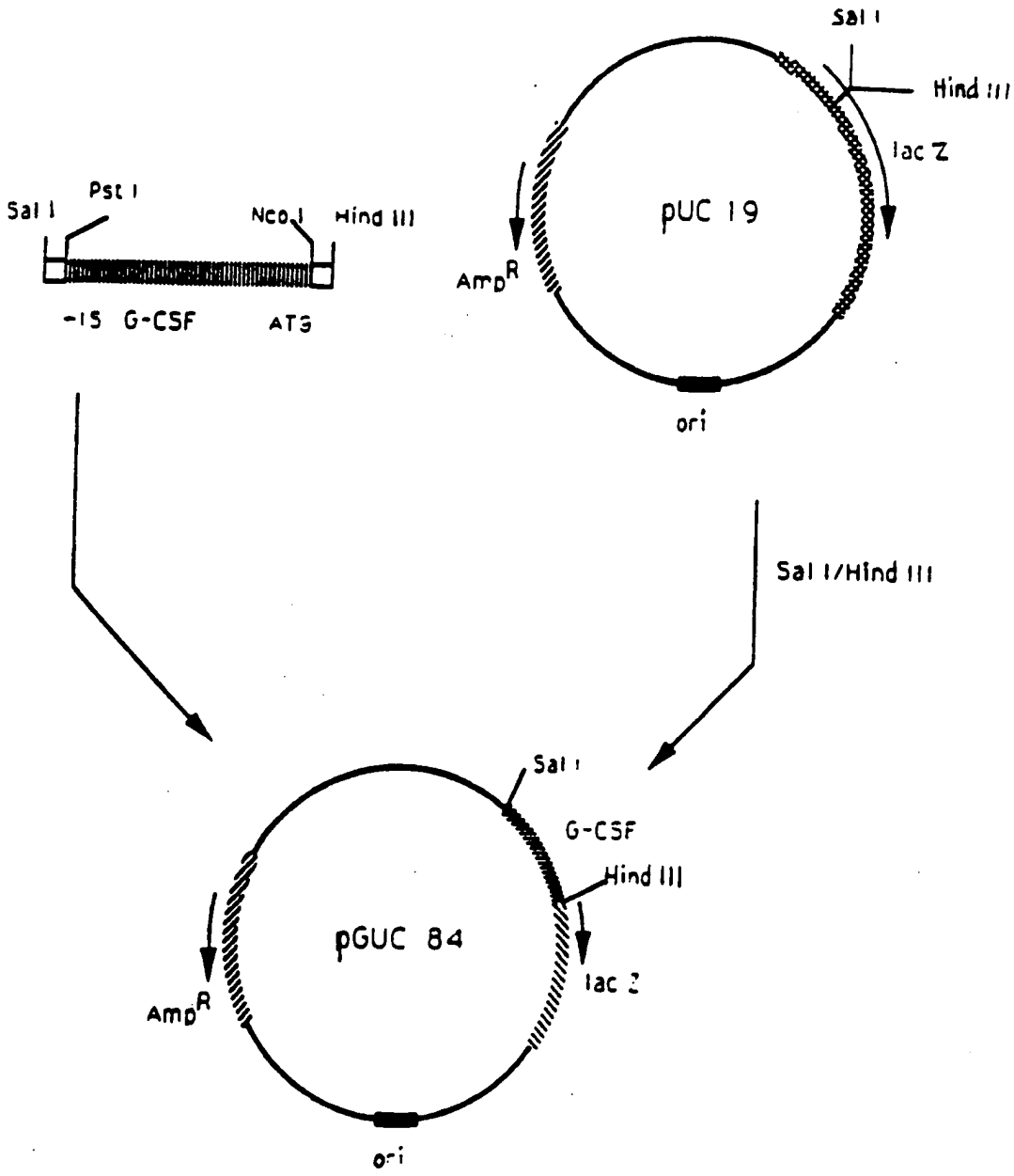


FIGURE 19. CONSTRUCTION OF pGUV 100

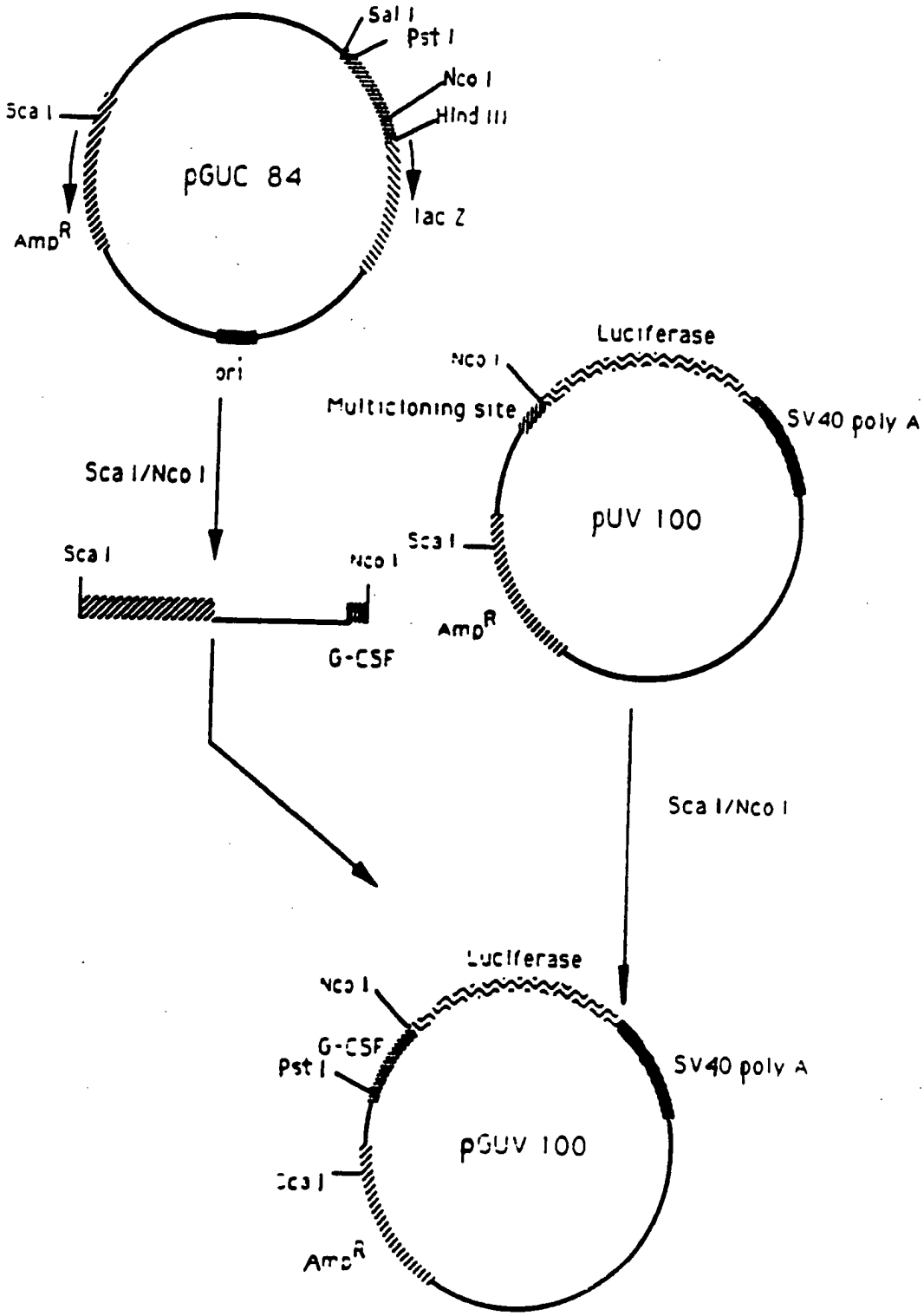


FIGURE 20. CONSTRUCTION OF pGUV 1

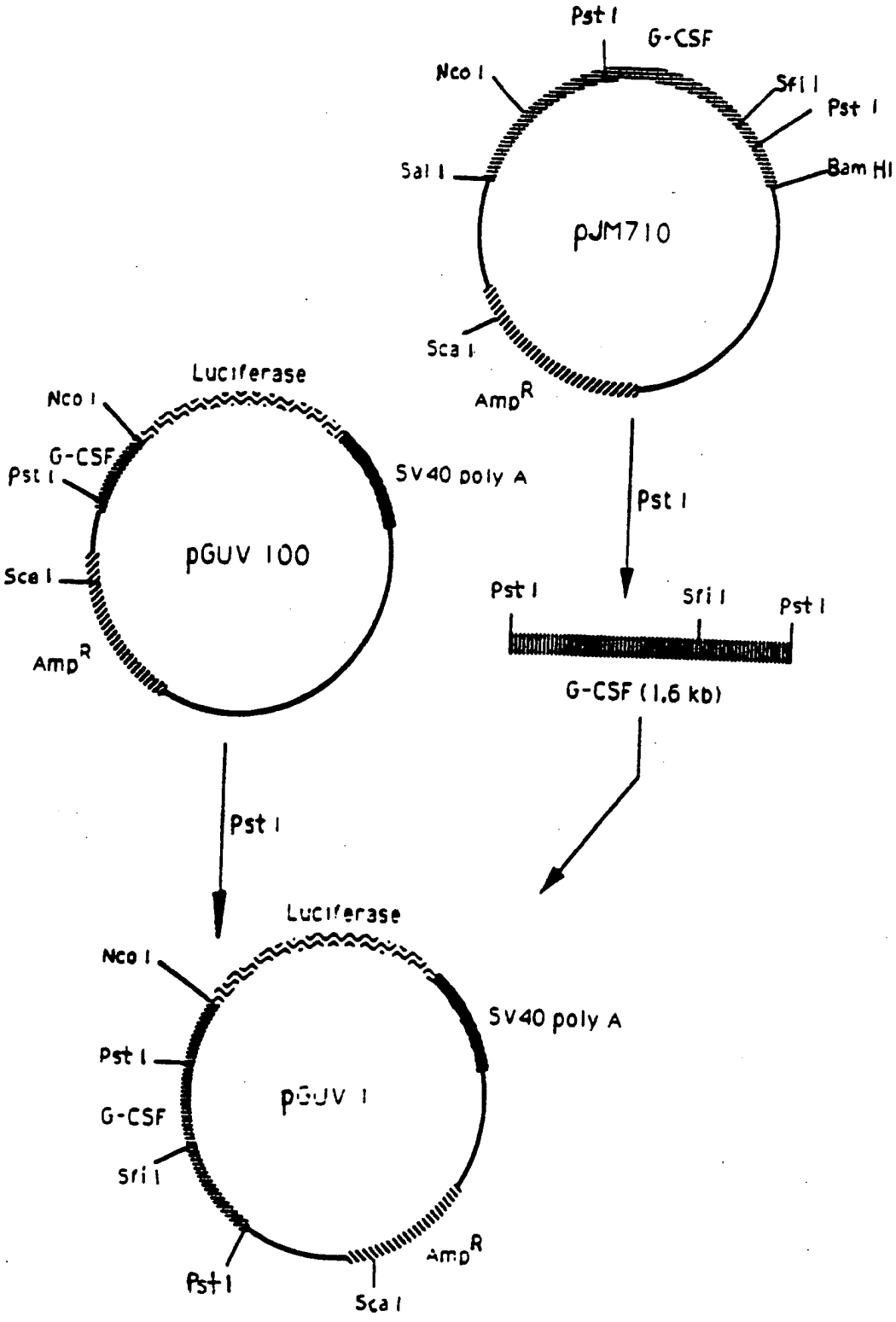


FIGURE 21. CONSTRUCTION OF pGUV 2

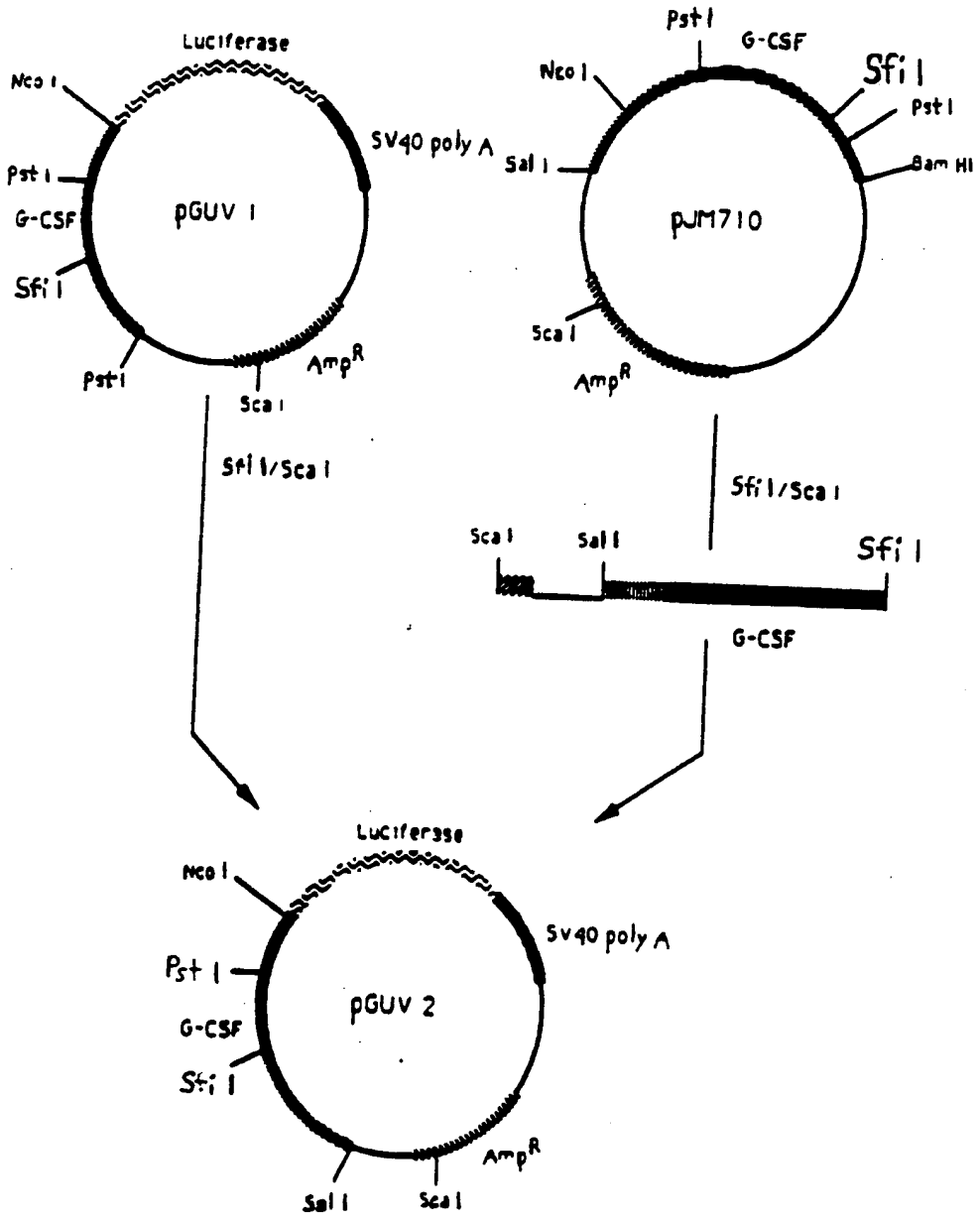


FIGURE 22. CONSTRUCTION OF pGUV 140

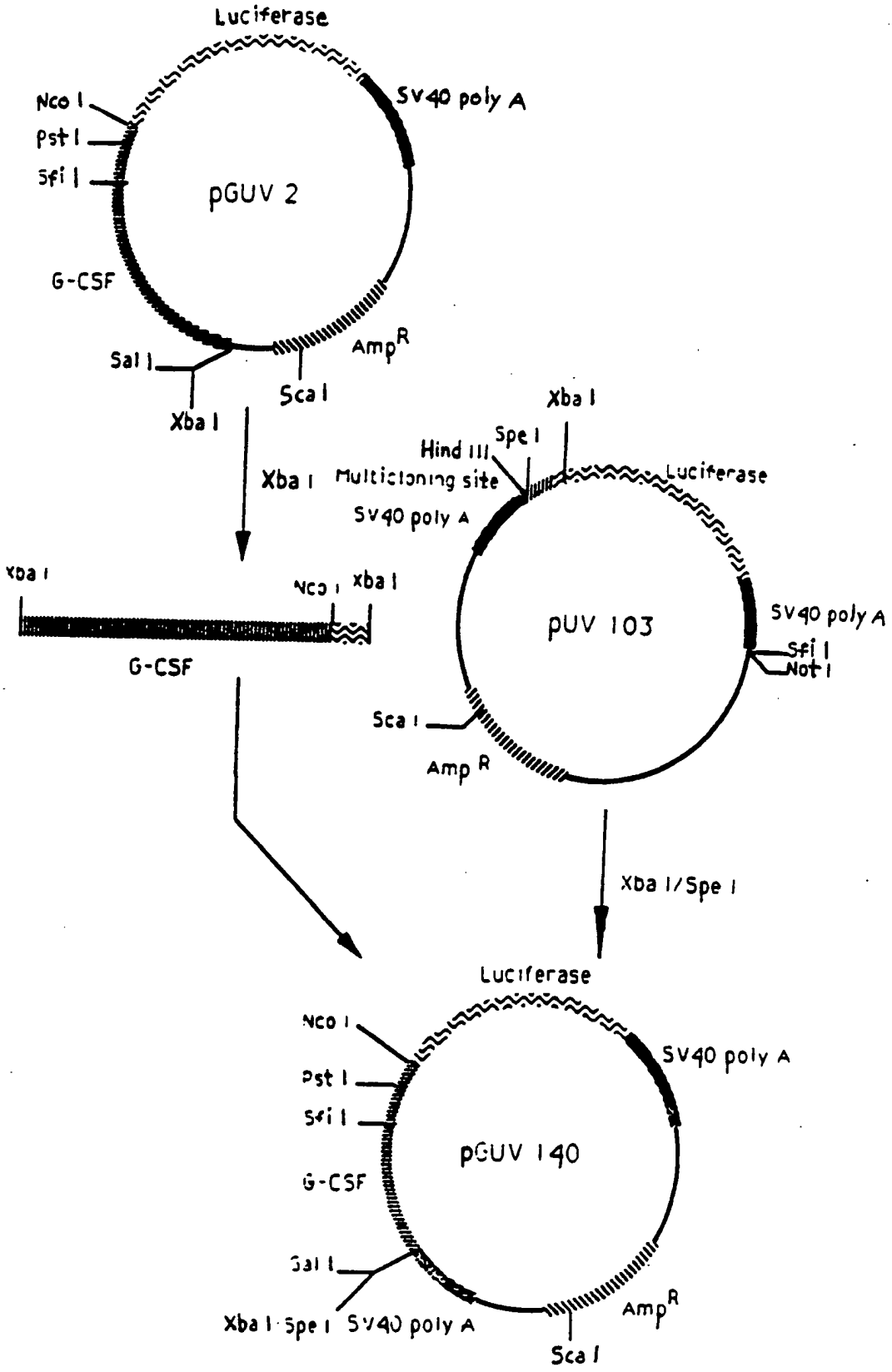


FIGURE 23. CONSTRUCTION OF pGUV 150

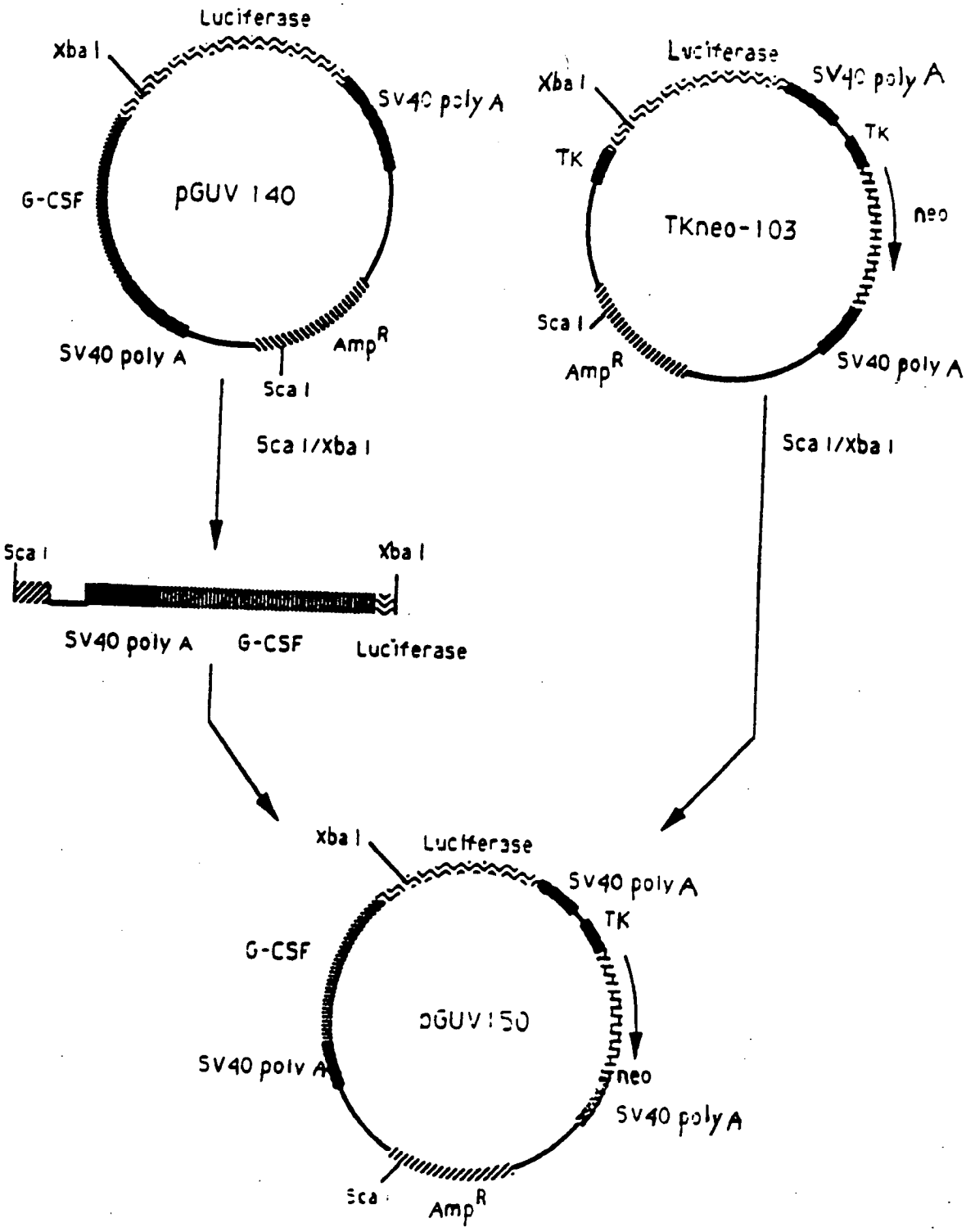
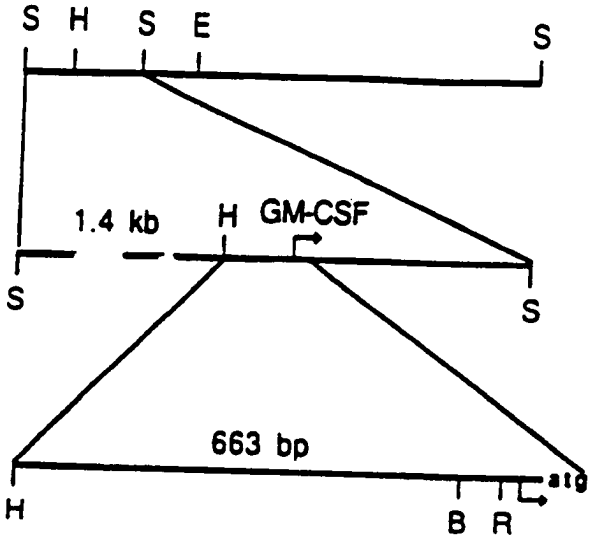


FIGURE 24. STRUCTURE OF THE GM-CSF CLONE



Key

S= Sal I
H= Hind III
E= Eco RI
B= Bst EII
R= Rsa I
↱ = start of transcription

FIGURE 25. CONSTRUCTION OF pGMLS102 AND pGMLS103

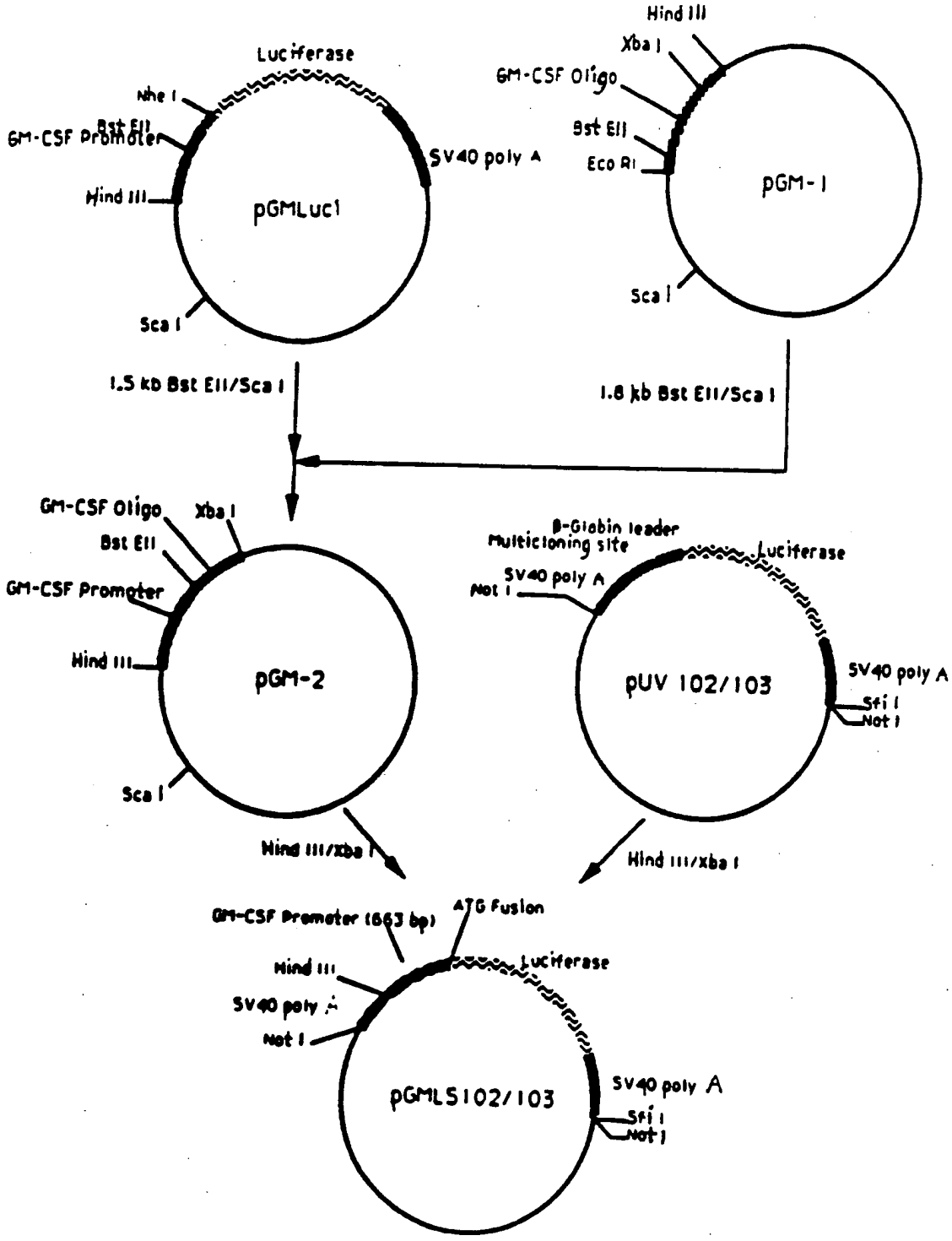


FIGURE 26. SYNTHETIC OLIGO FOR GM-CSF/LUCIFERASE ATG FUSION

Eco RI ends Bsl E II

OLIGO 1 5' AATTCGGTCACCATTAATCATTTCCTCTGTGTATTTAAGAGCTCTTTTGCCAGTGAGCCCAGTACACAG 3'
OLIGO 2 3' GCCAGTGGTAATTAGTAAAGGAGACACATAAATTCTCGAGAAAACGGTCACTCGGGTCATGTGTCTCTCTTTCCG 5'

ATG Xba I ends

OLIGO 3 5' AGAGAAAGGCTAAGTTCTCTGGAGGATGGAAGACGCCAAAAACATCAAGAAAGGCCCGGCGCCATTCTATCCT 3'
OLIGO 4 3' ATTTCAAGAGACCTCCTACCTTCTGCGGTTTTTGTAGTTCTTTCCGGGCCGCGGTAAGATAGGAGATC 5'

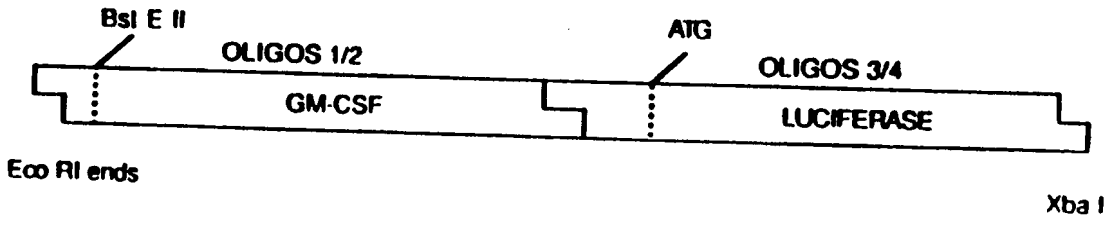


FIGURE 27. CONSTRUCTION OF pGMLL102 AND pGMLL103

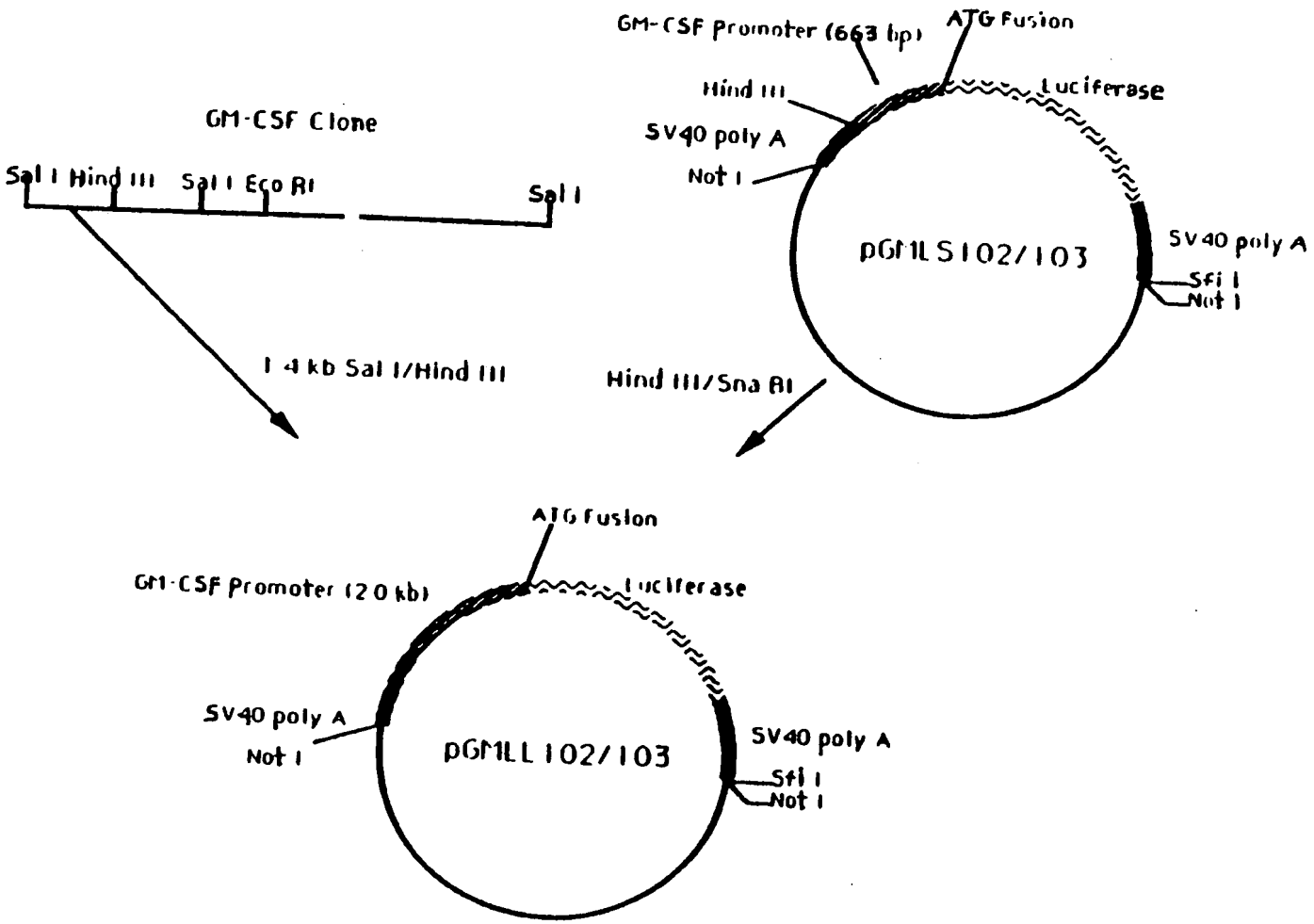


FIGURE 28. CONSTRUCTION OF CSF1 - pTZ18

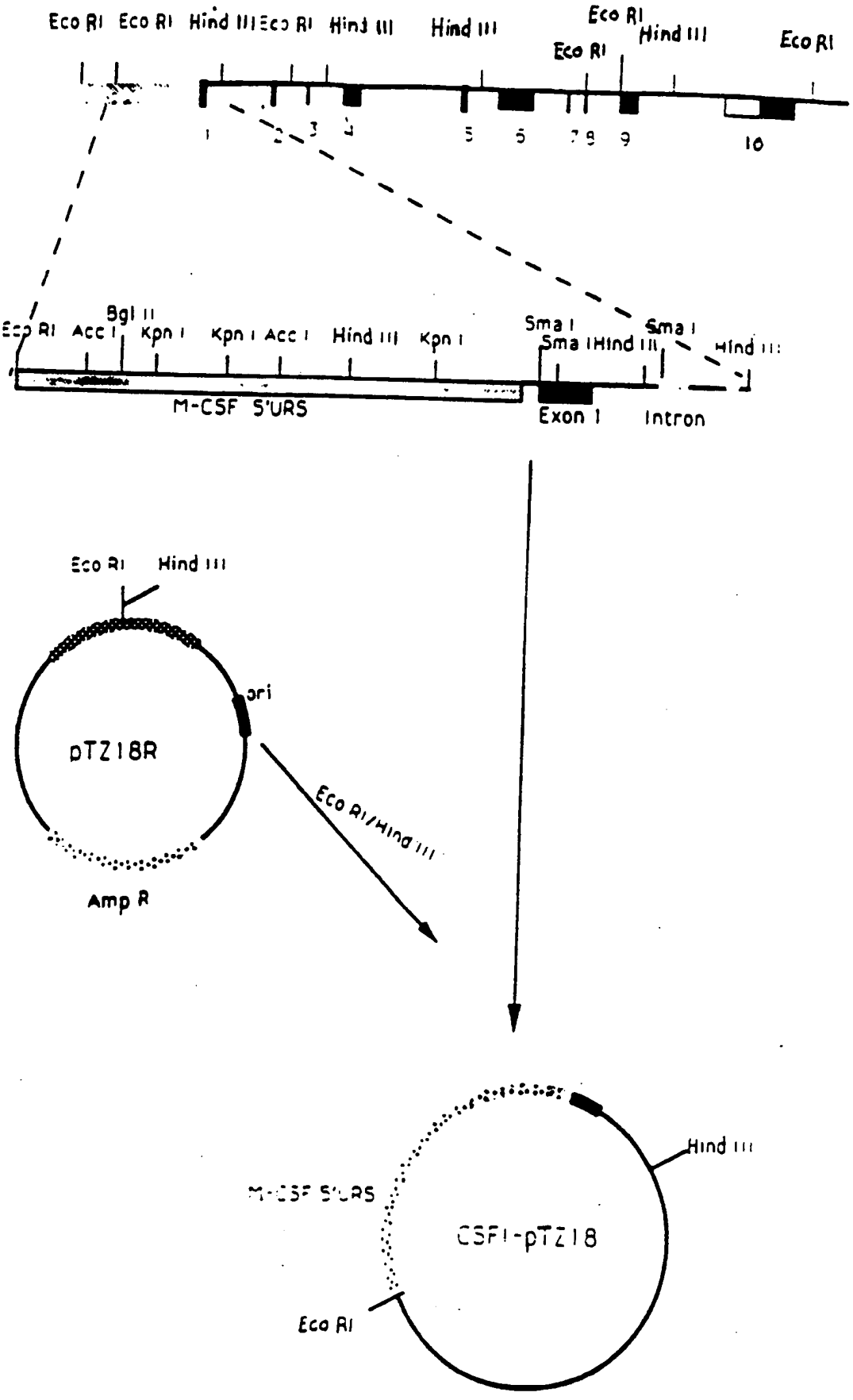


FIGURE 29. CONSTRUCTION OF p100 - RH

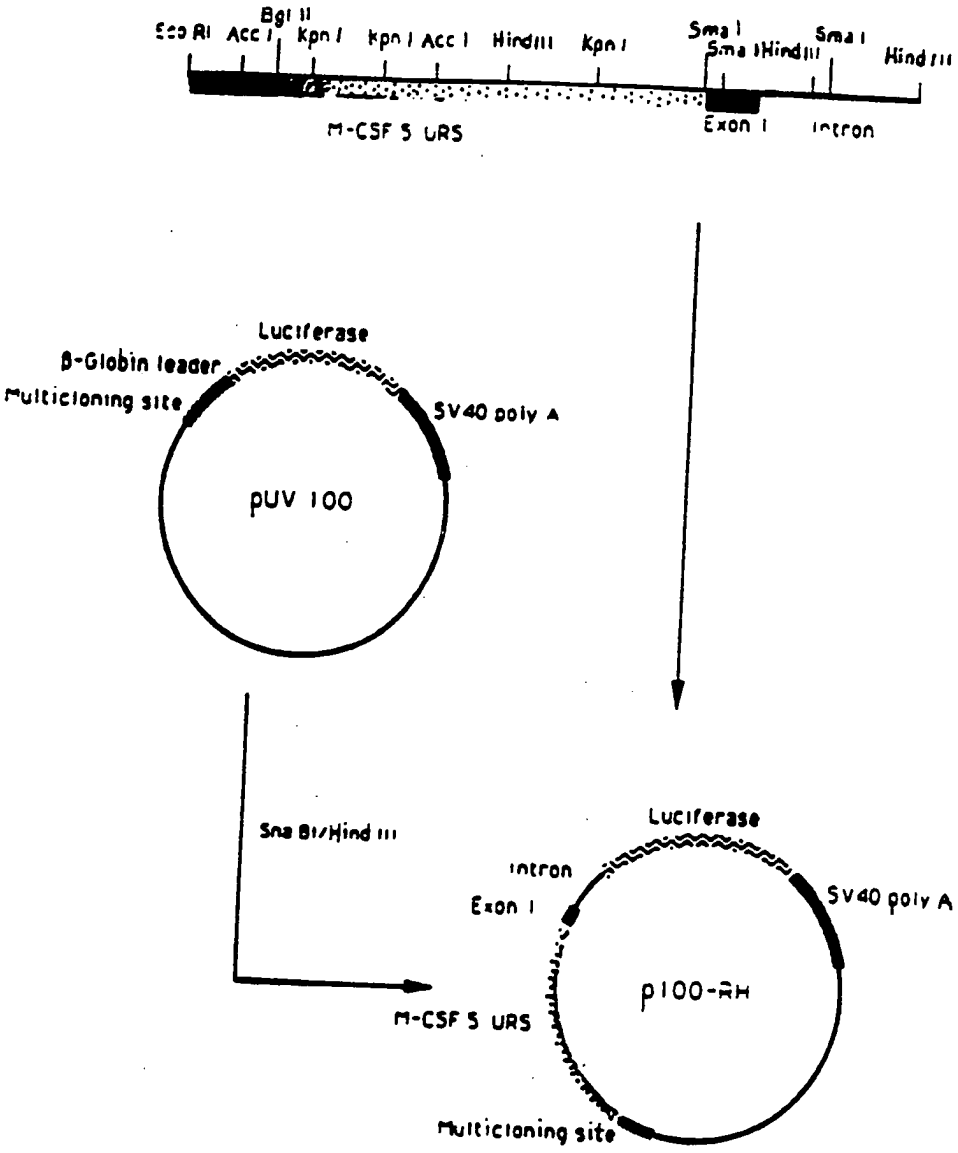


FIGURE 30. CONSTRUCTION OF p100 - 2

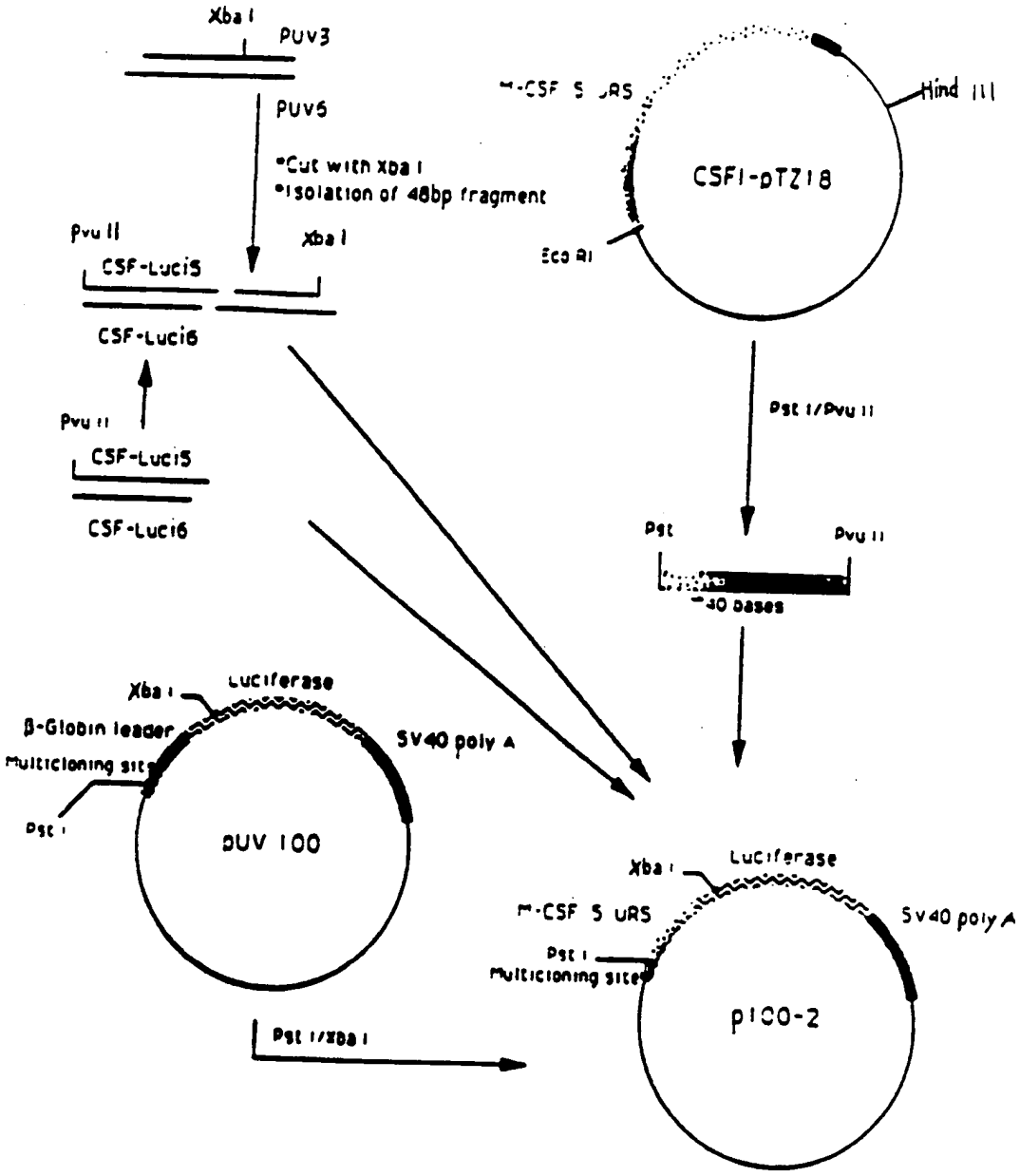


FIGURE 31. CONSTRUCTION OF p100 - RHC

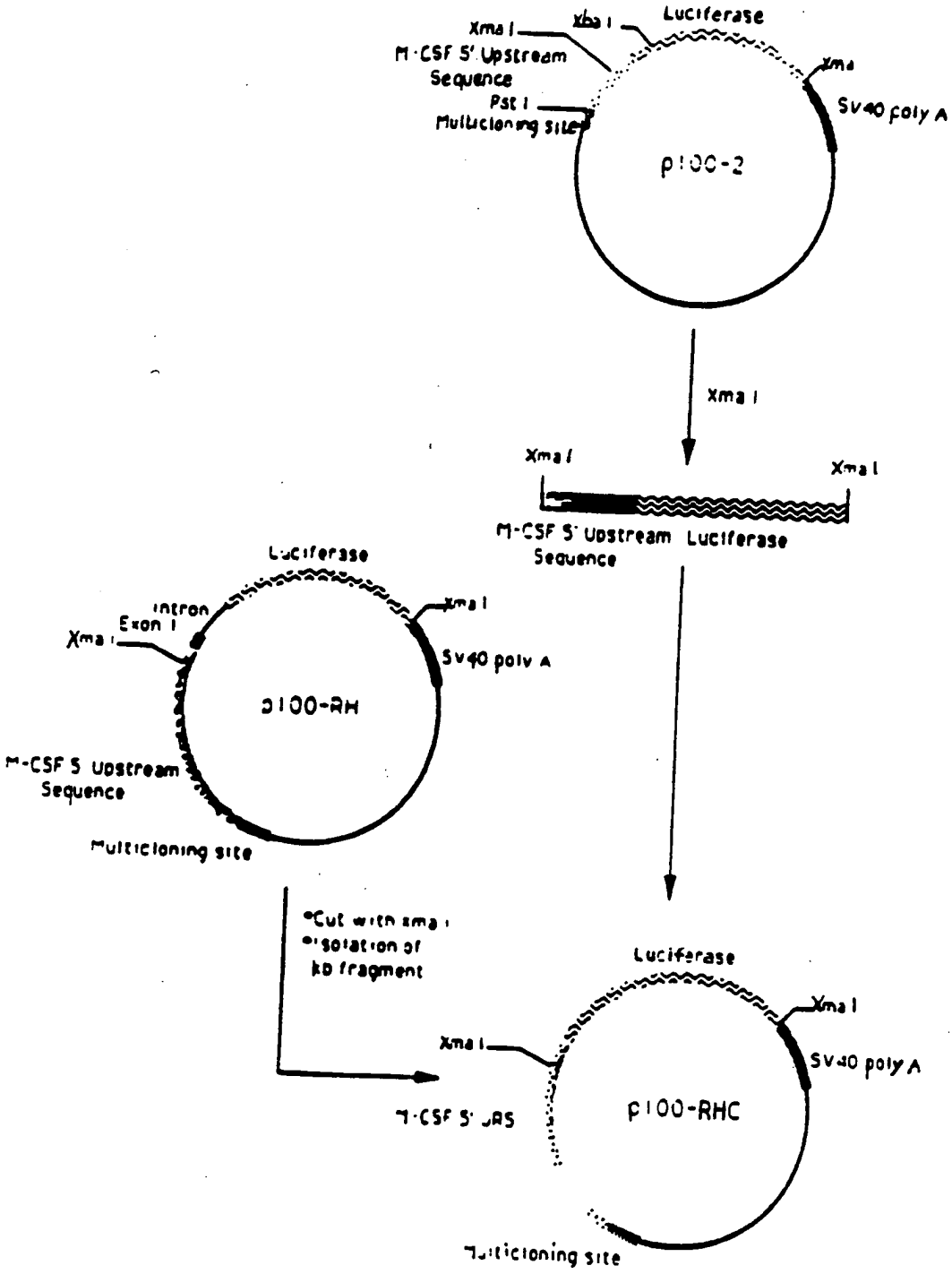


FIGURE 32. CONSTRUCTION OF pCSF1 - 102

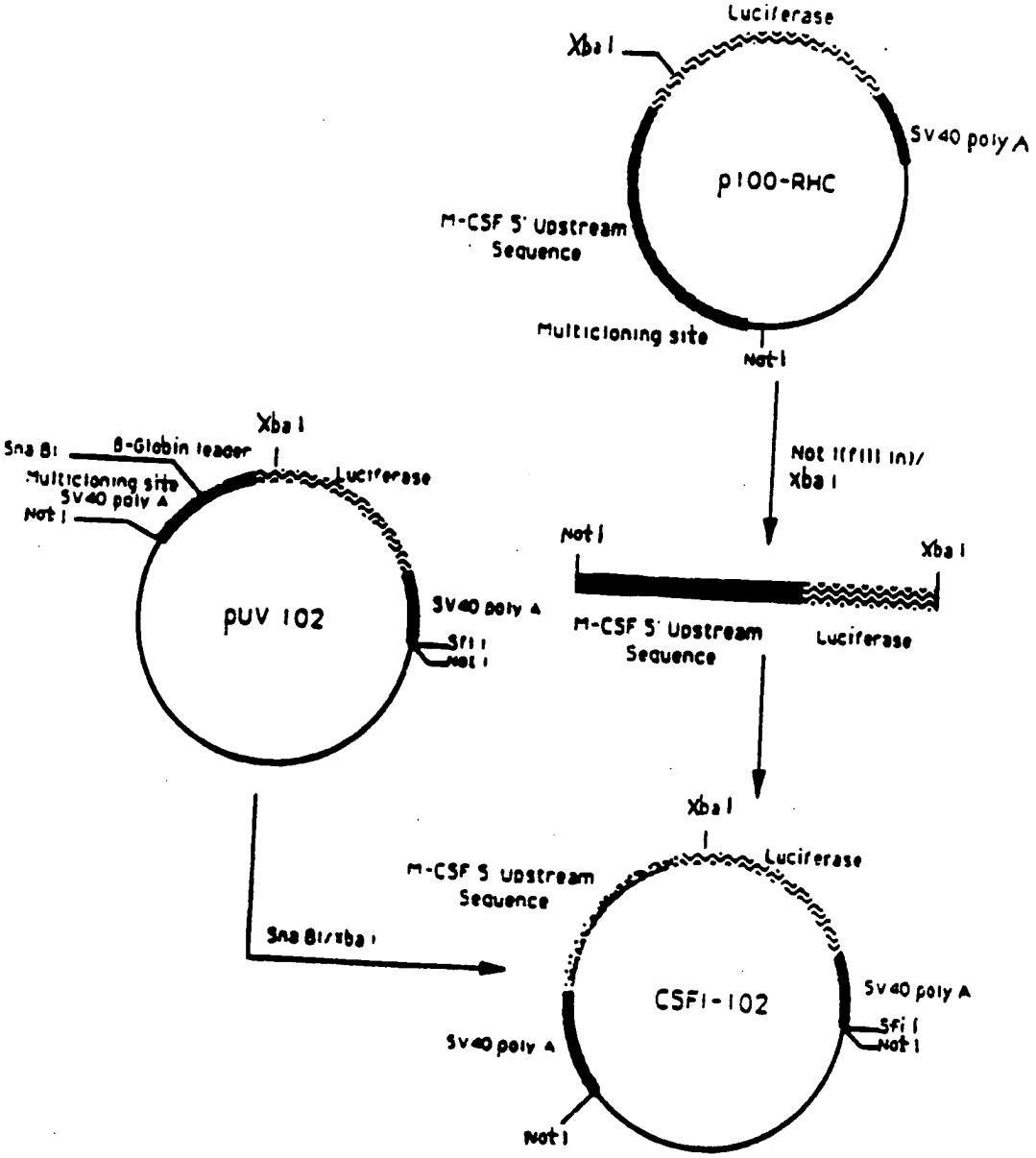


FIGURE 33. CONSTRUCTION OF pEP - 7.5B

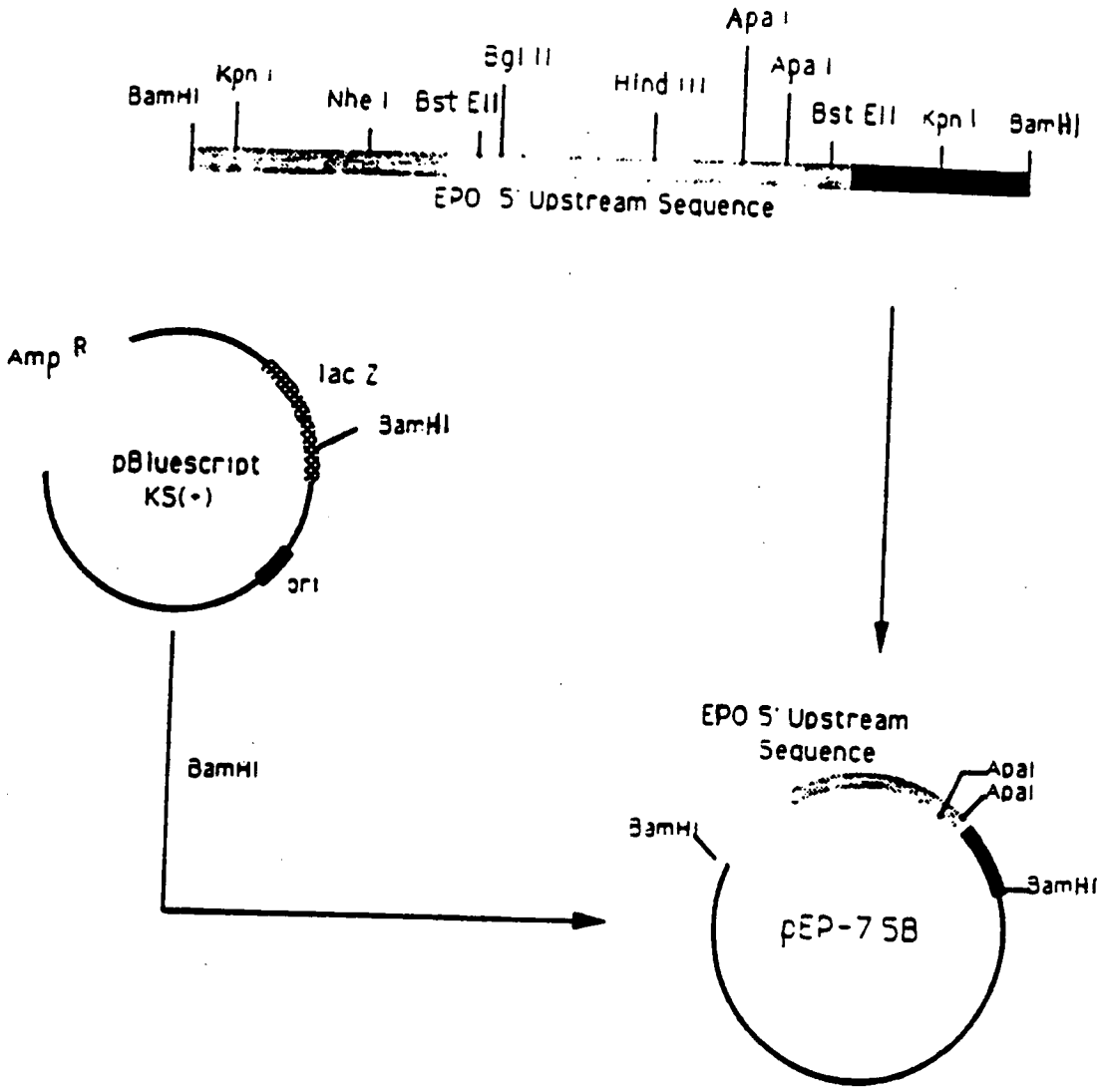


FIGURE 34. CONSTRUCTION OF pRE

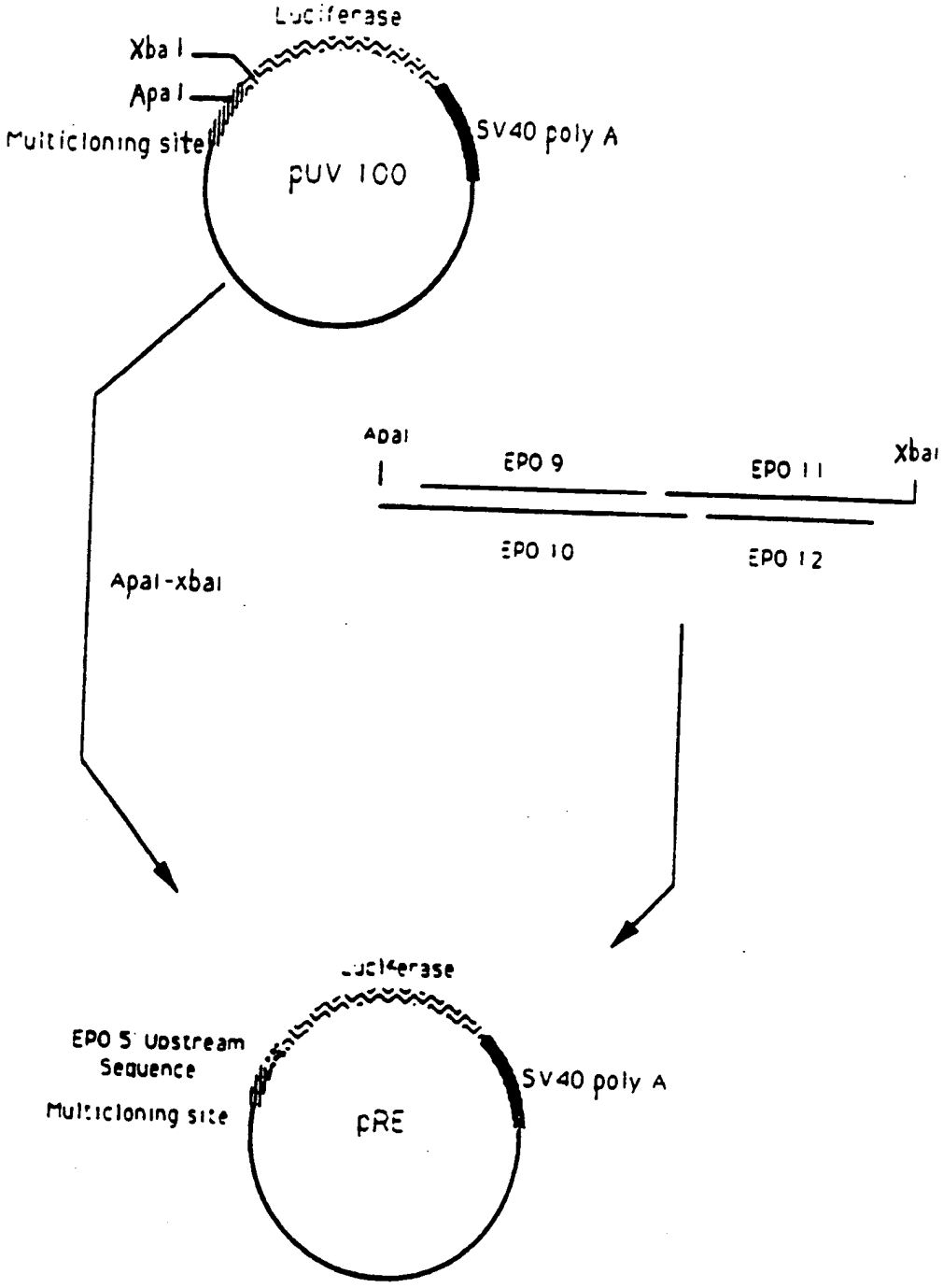


FIGURE 35. CONSTRUCTION OF pUV2 - EP6

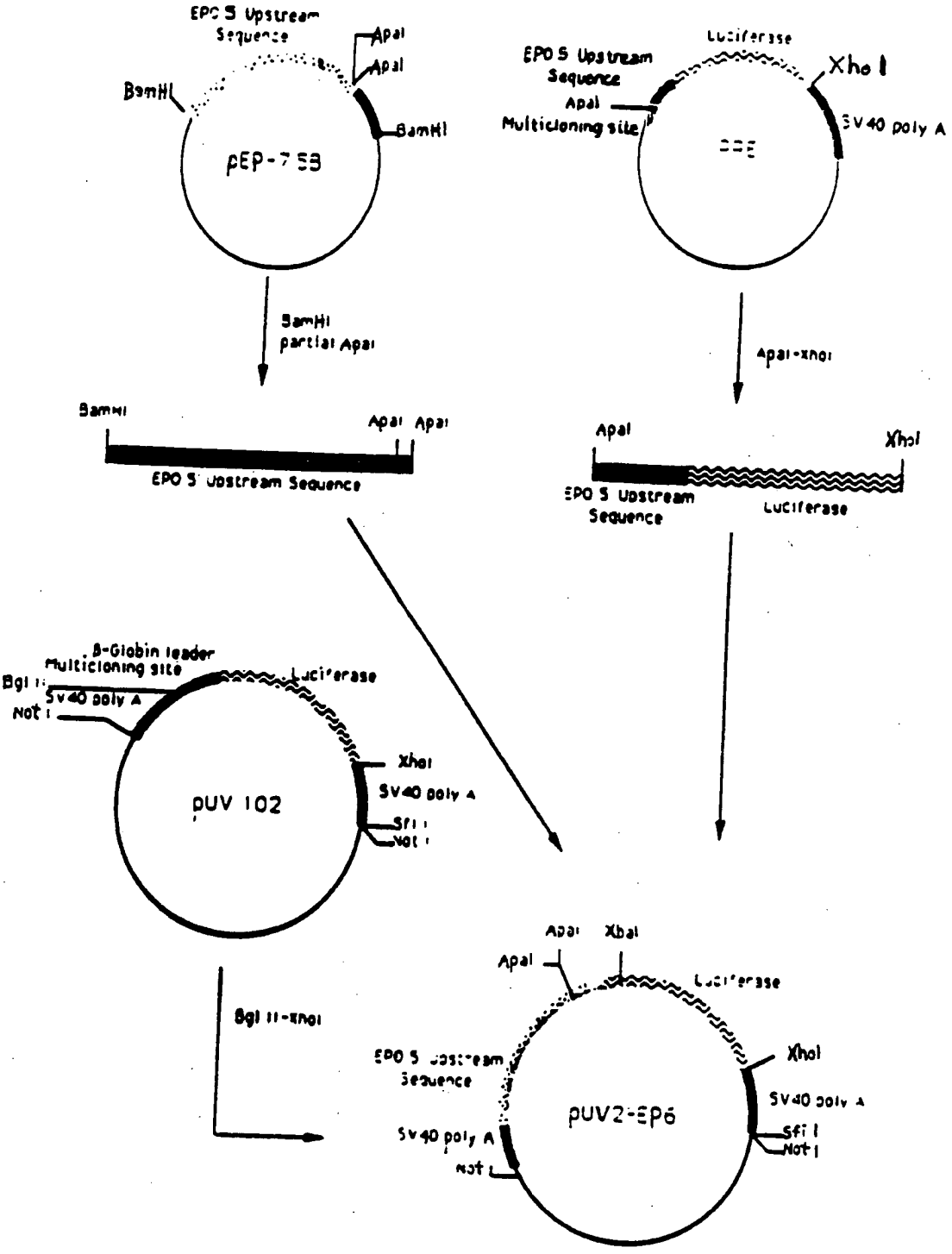


Figure 36

The Structure of pEPORF 106

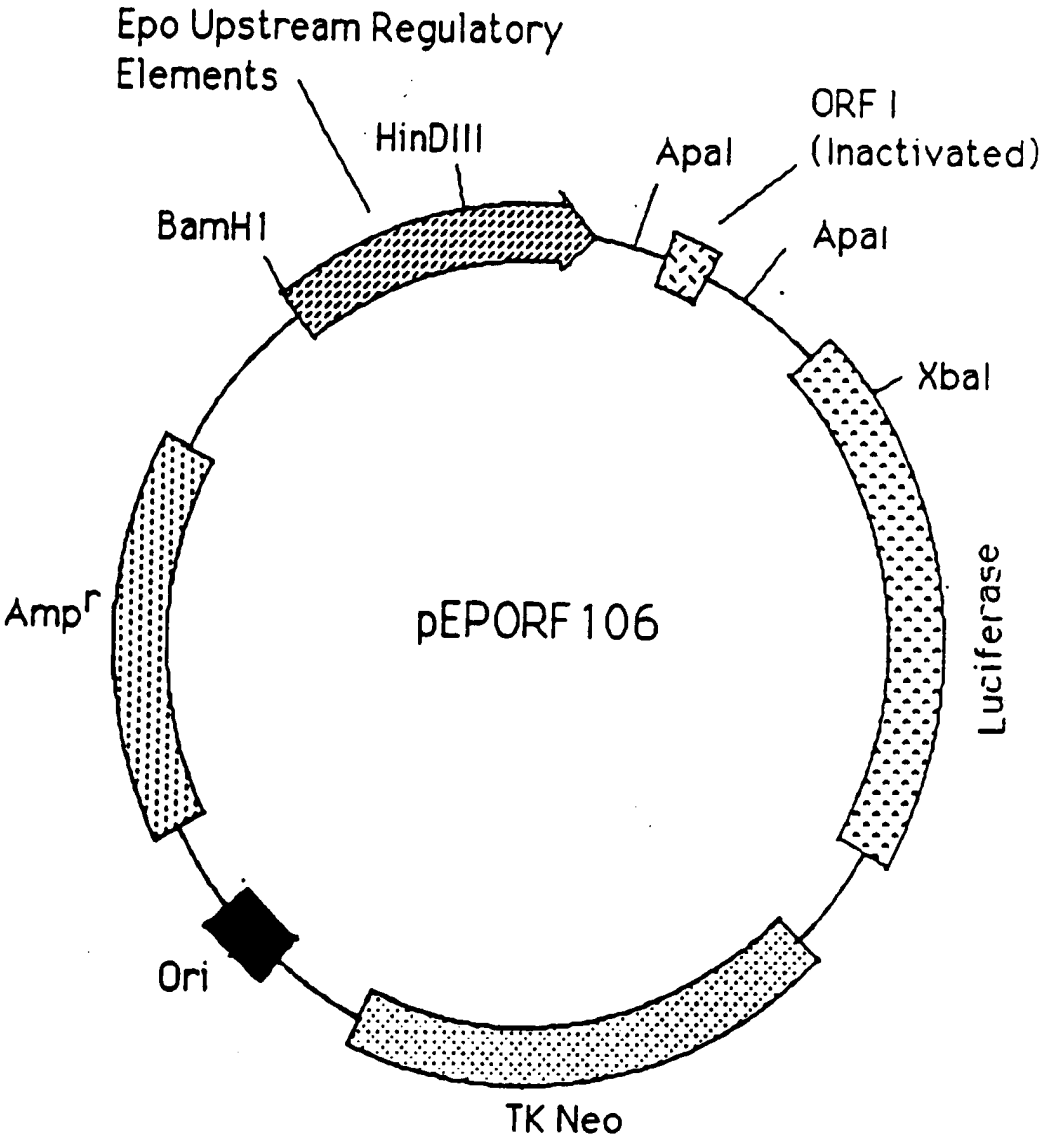


Figure 37

The Structure of pEPOEX106

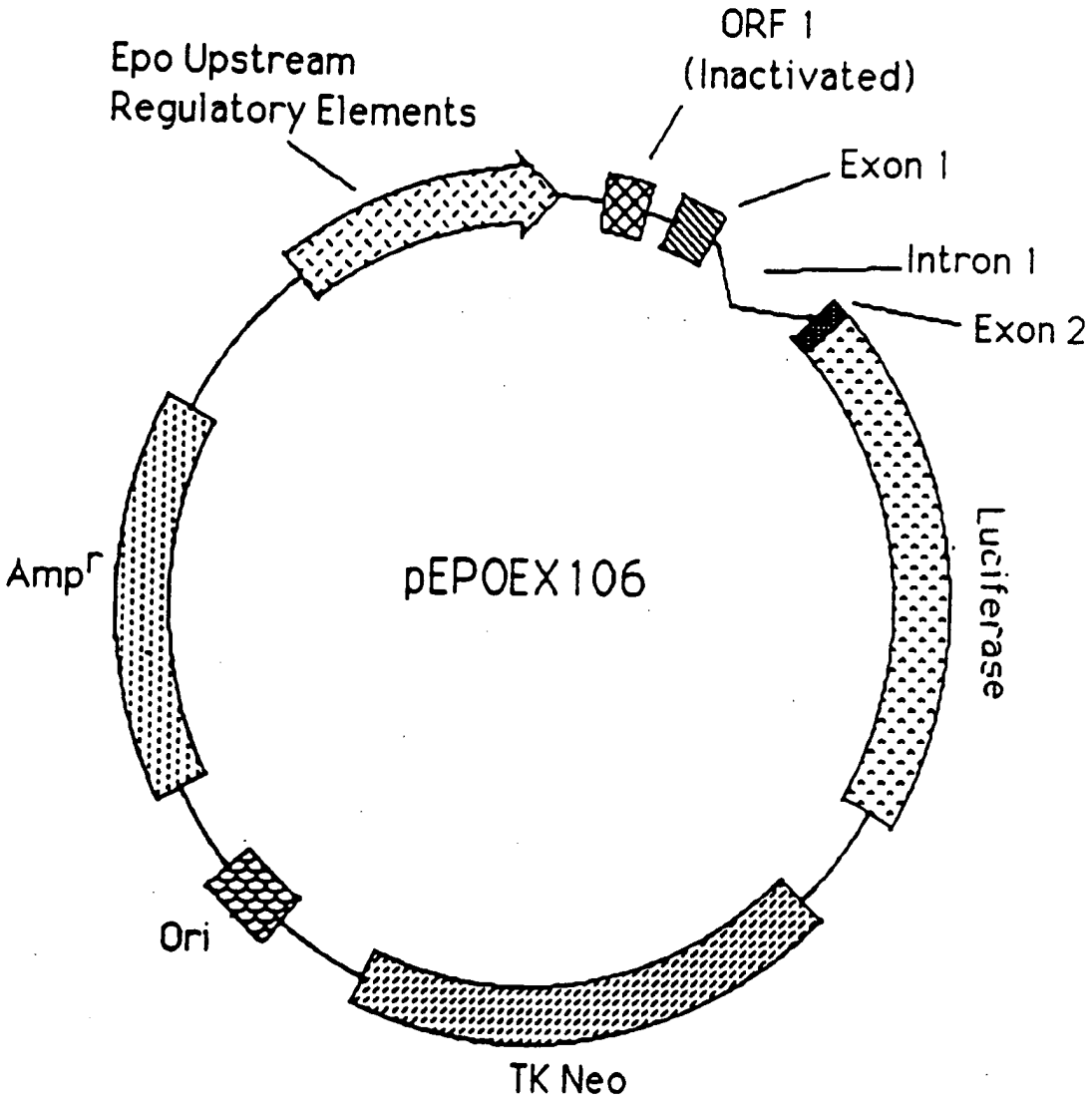


Figure 38. Construction of IL-3-pTZ18R

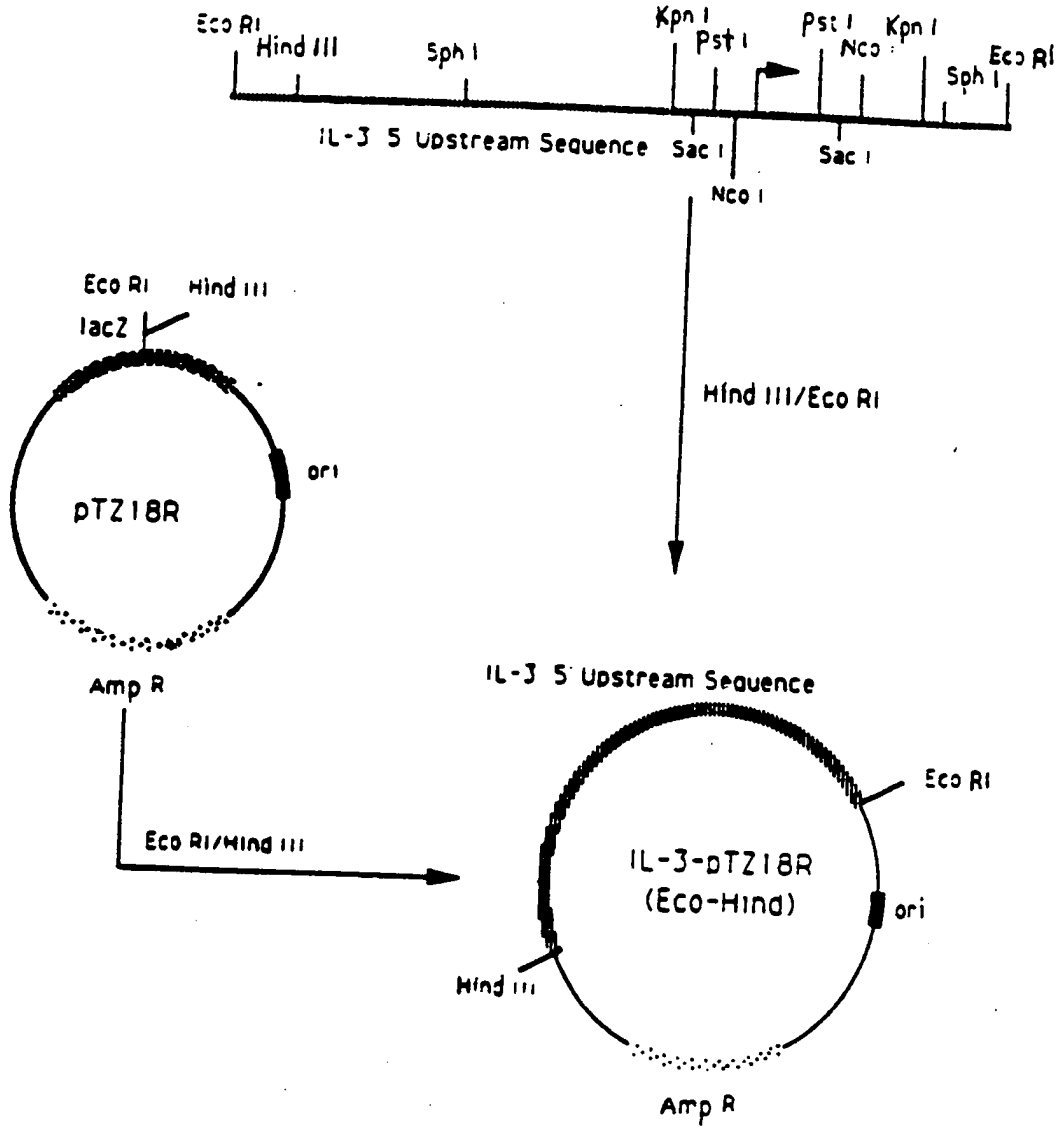


Figure 39. Construction of pTZ-IL-3-Luc1

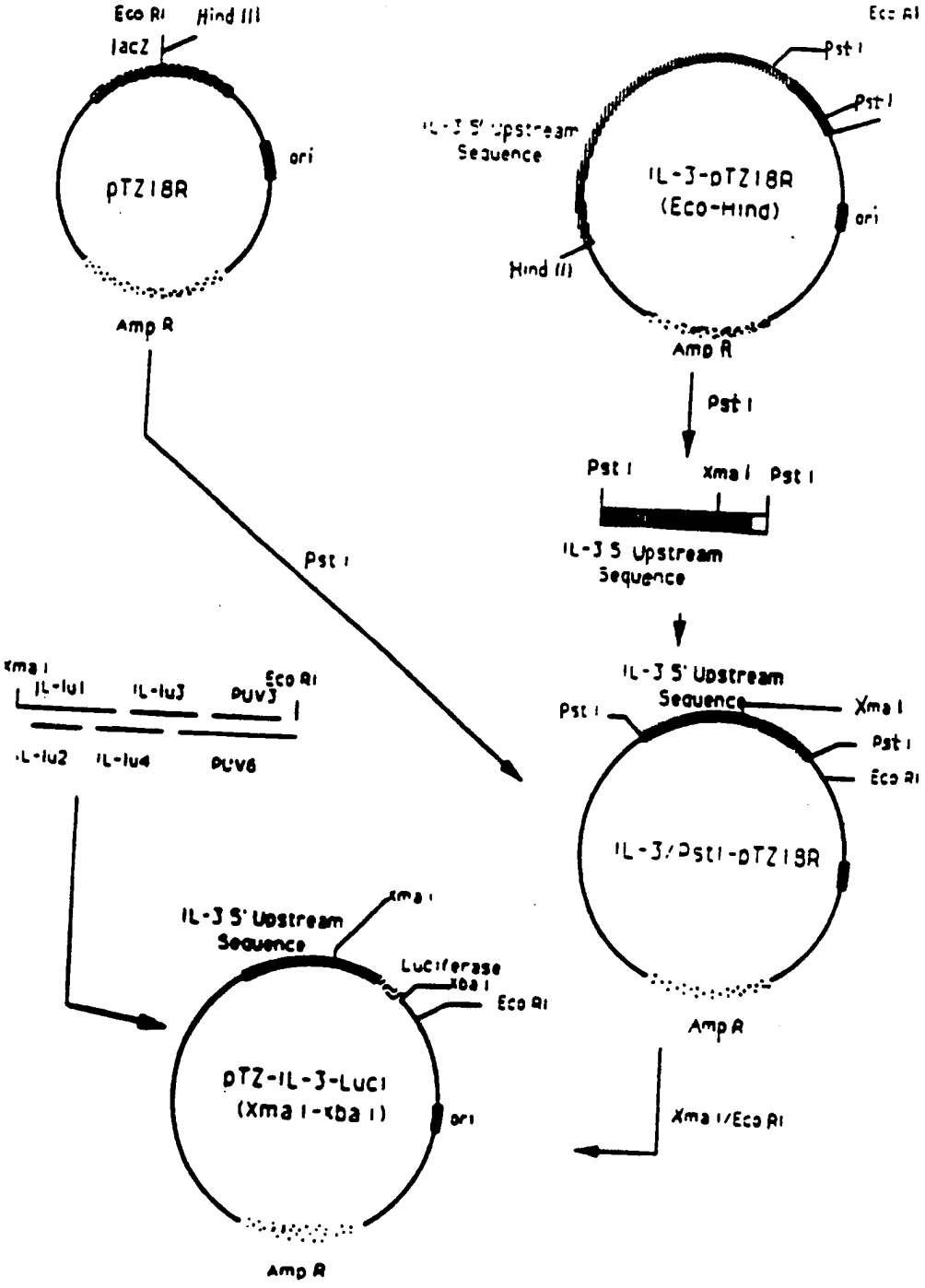


Figure 40. Construction of pTZ-IL3-C

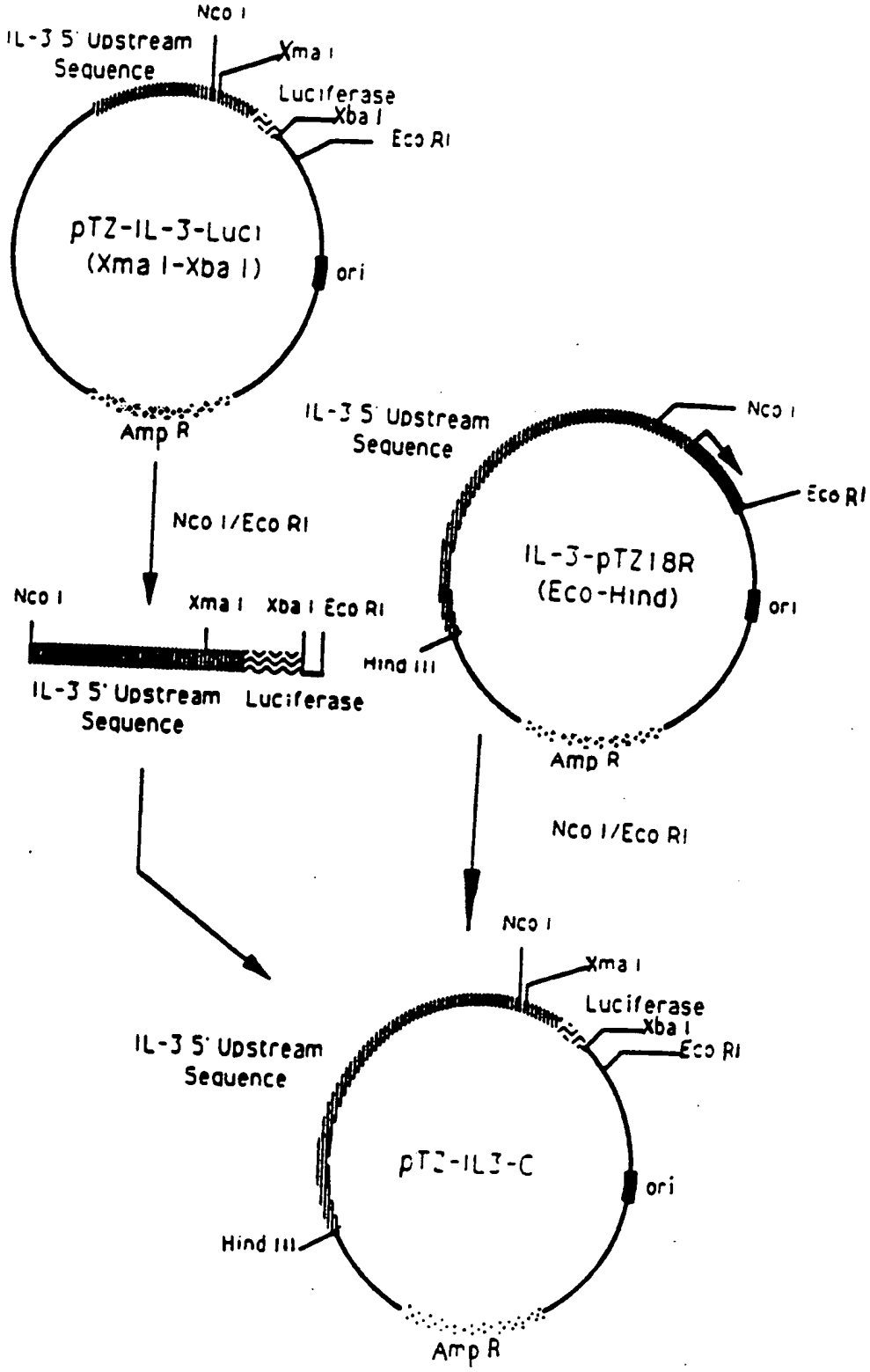


Figure 41 Construction of pUV-IL-3

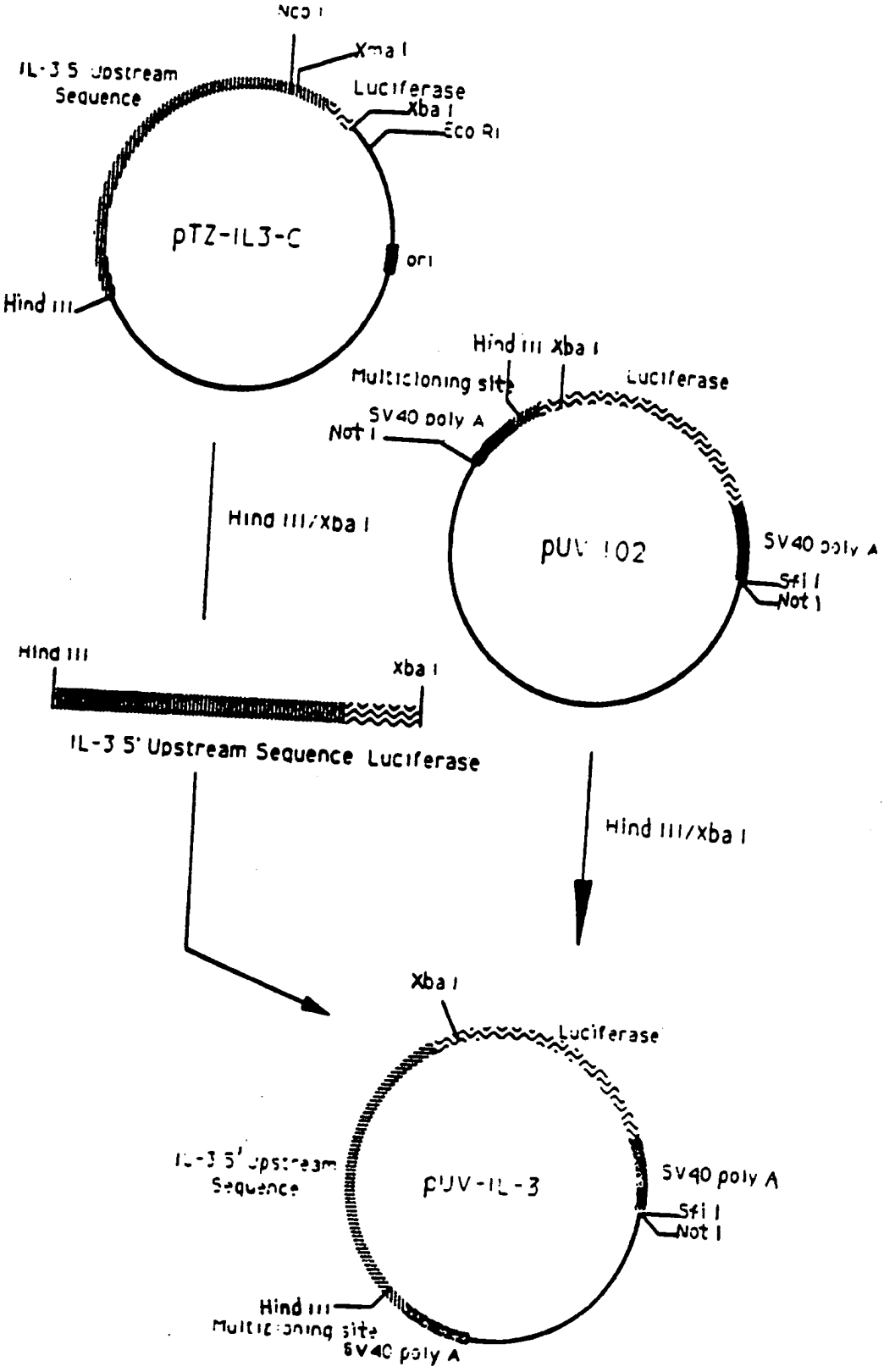


Figure 42

The Structure of pSCF106

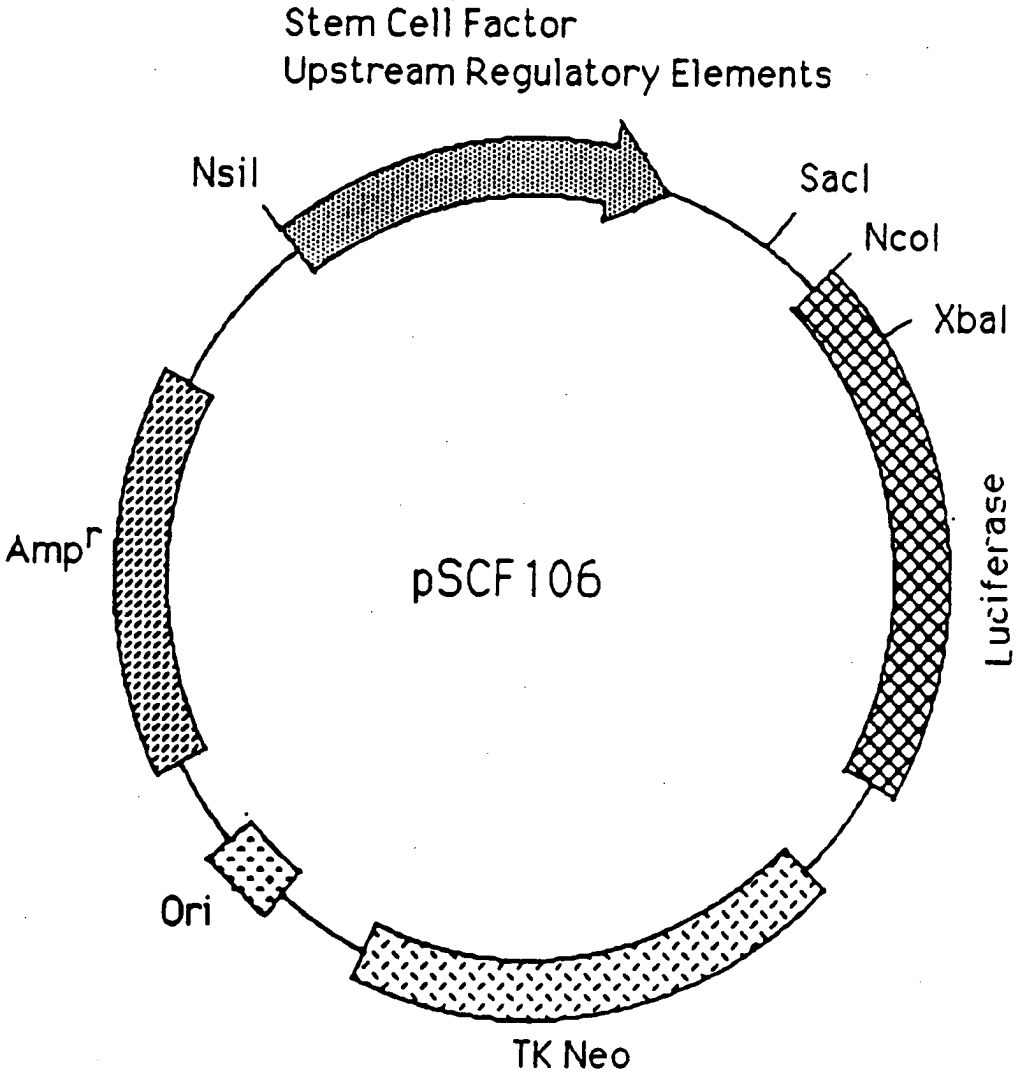


FIGURE 43. pUXLuci

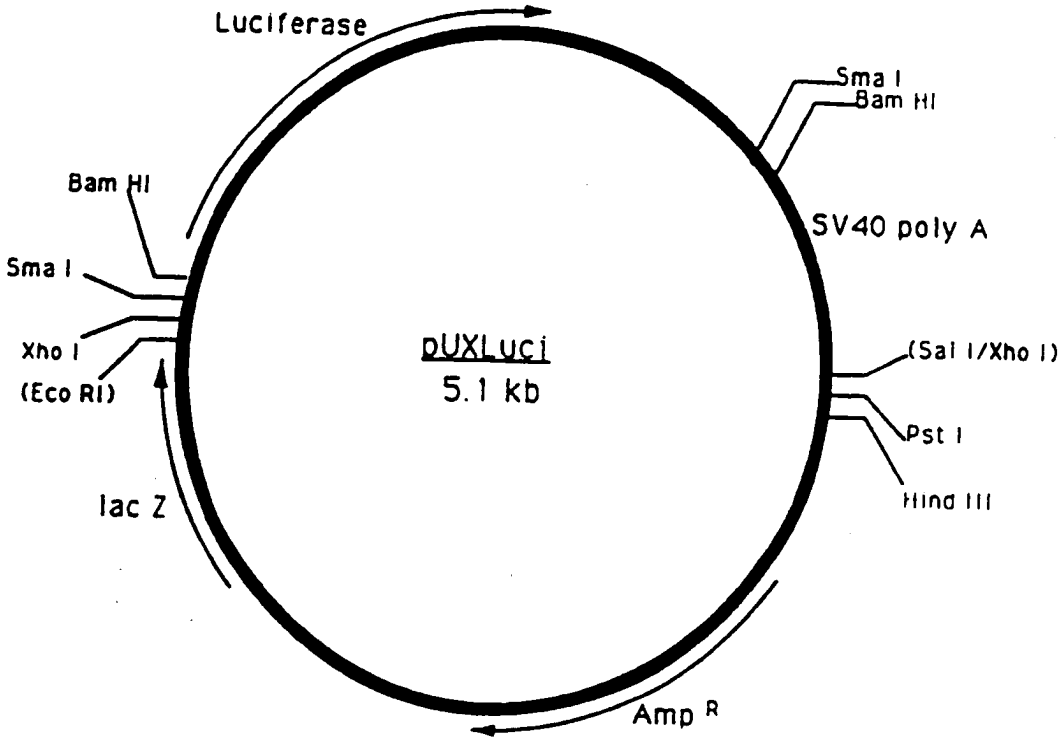


FIGURE 44. phGH - CAT

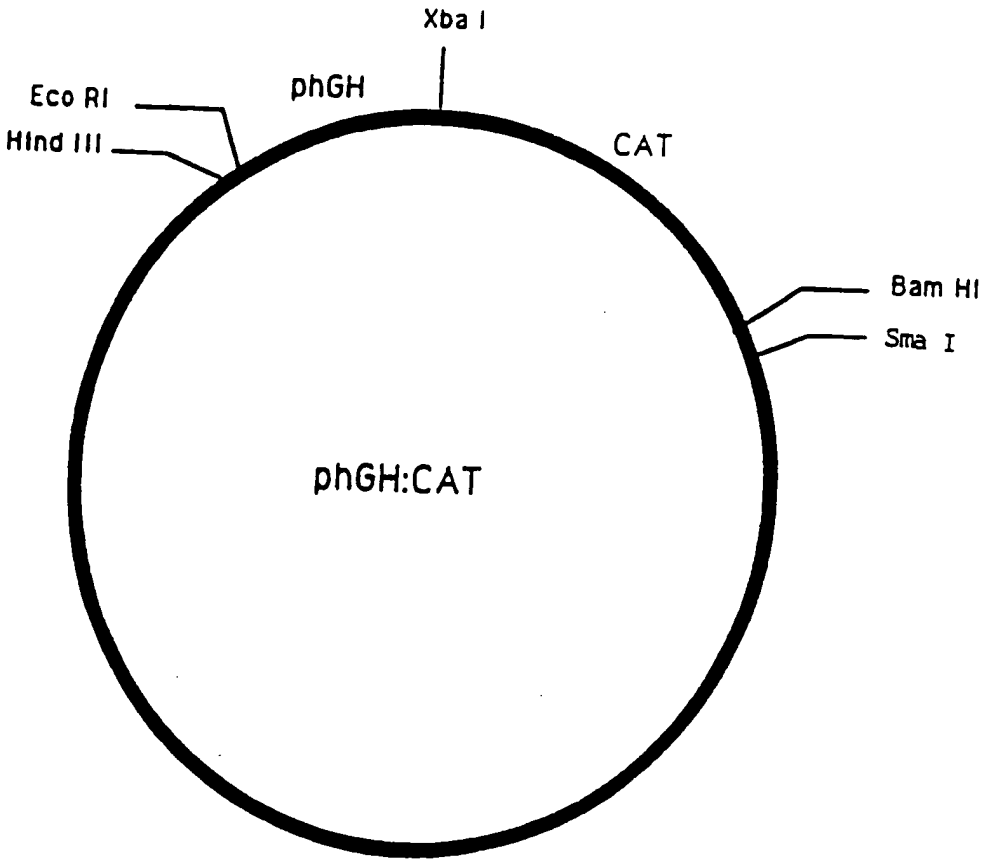


FIGURE 45. phGH - Luci

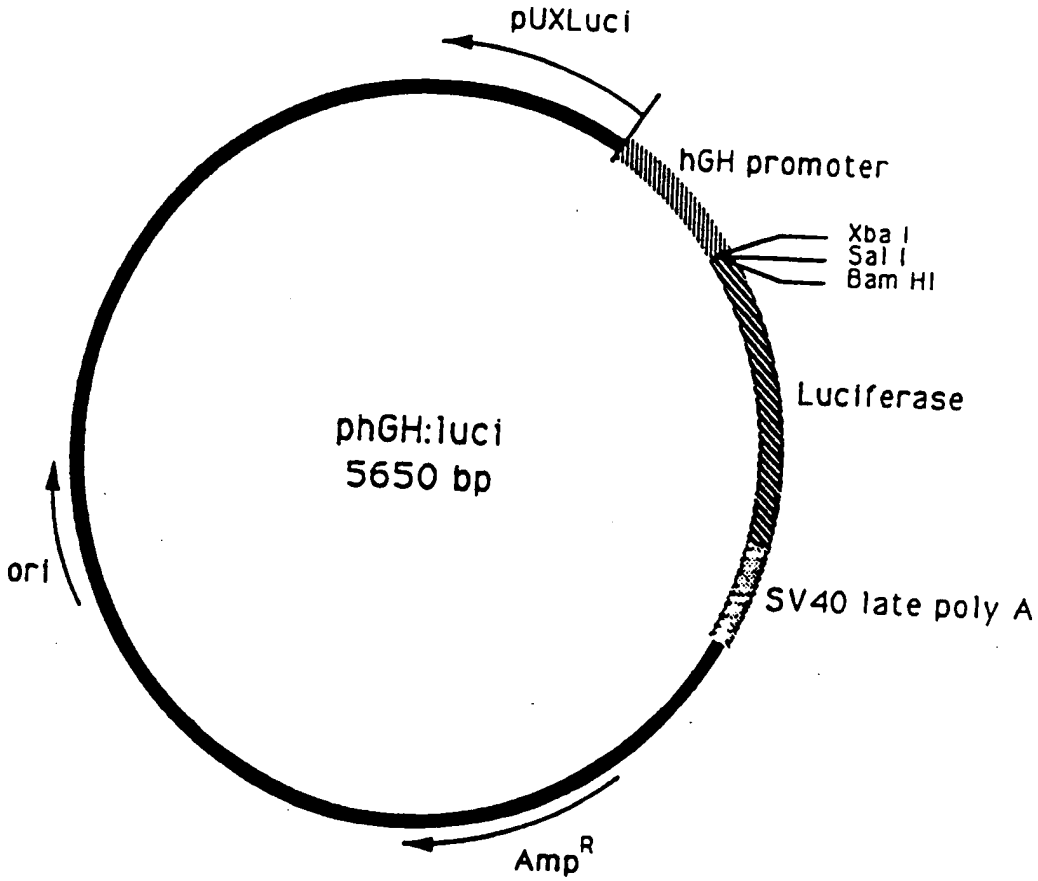


Figure 46
Quantitative PCR

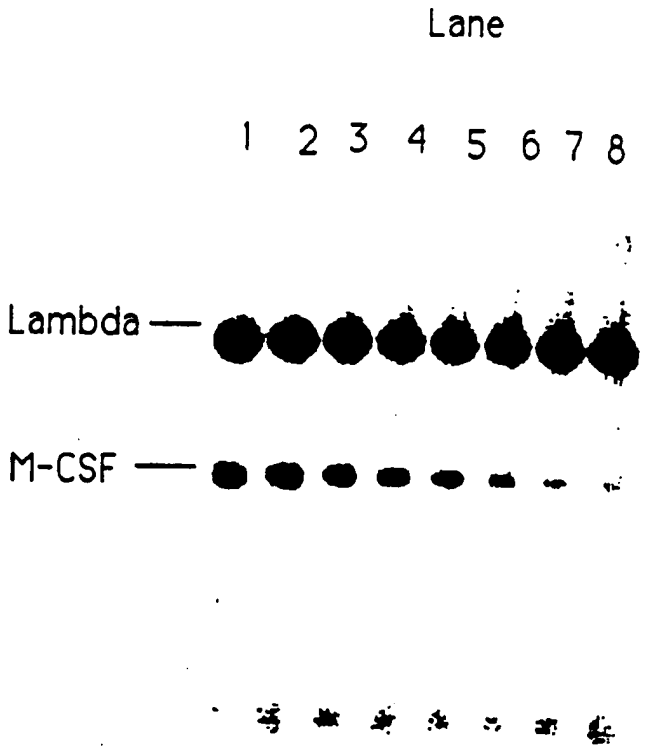


Figure 47
Quantitative PCR

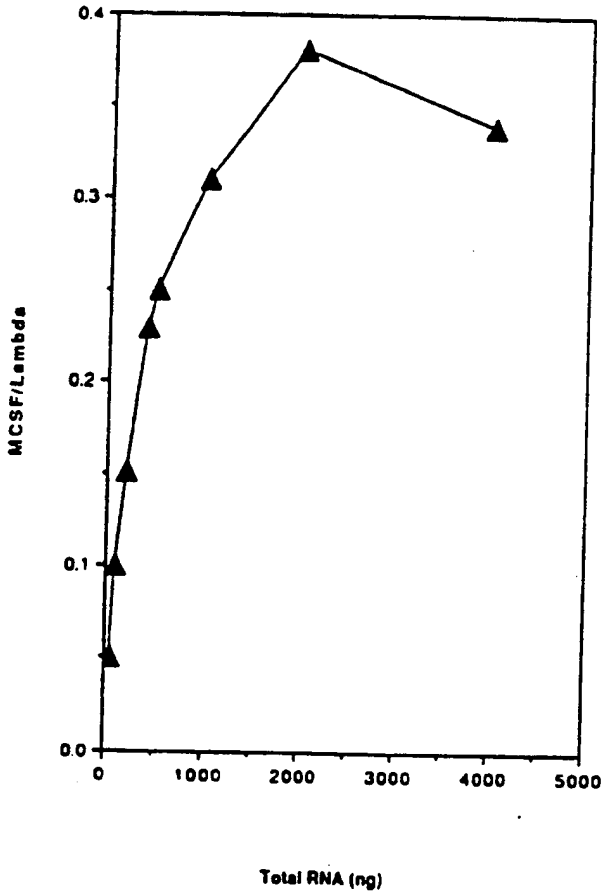


Figure 48

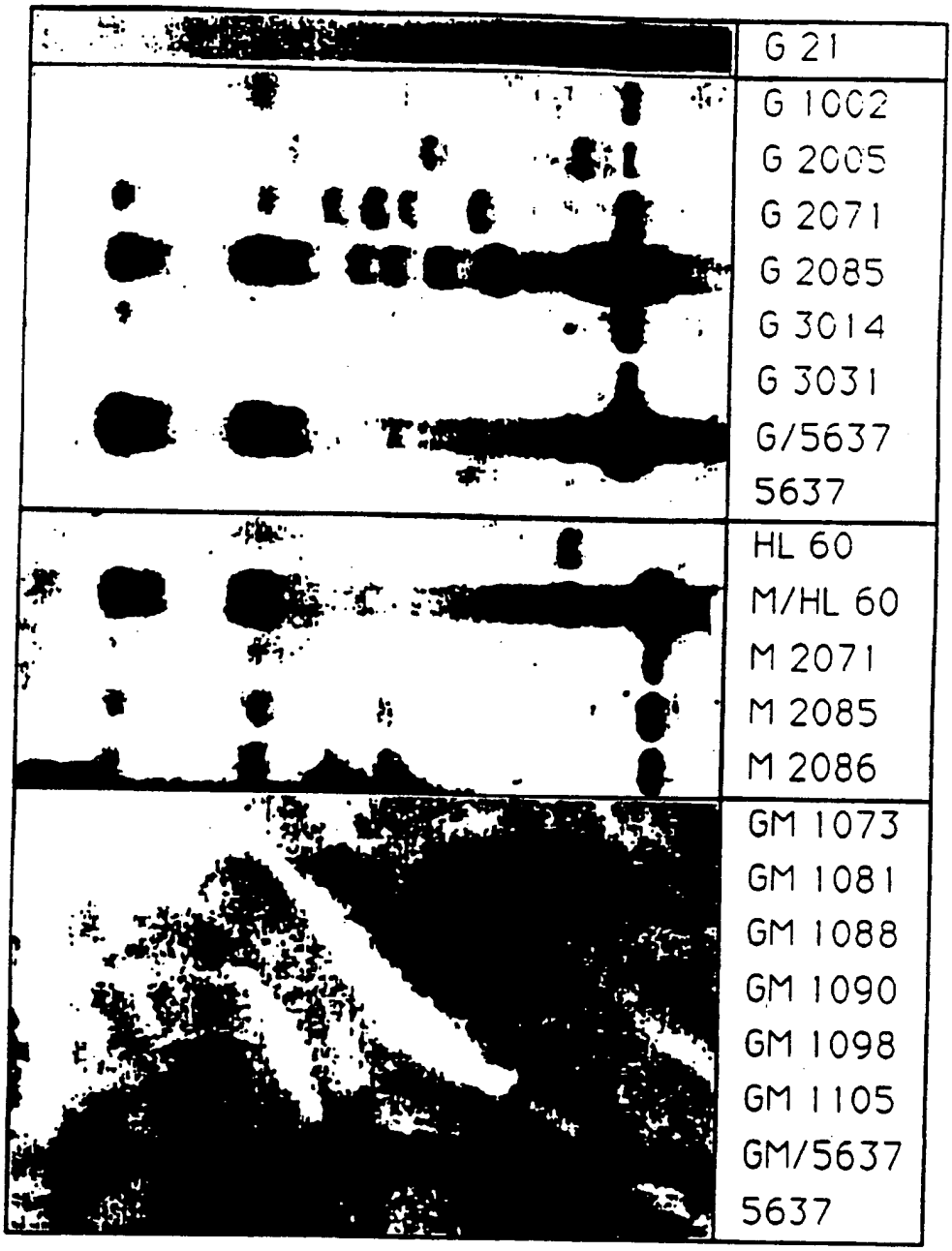
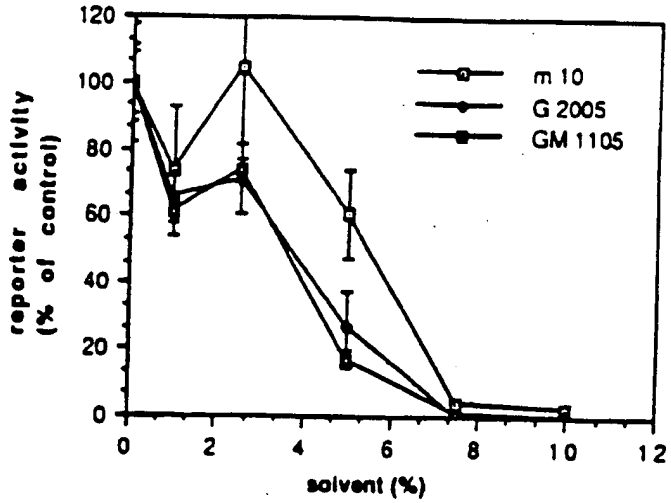
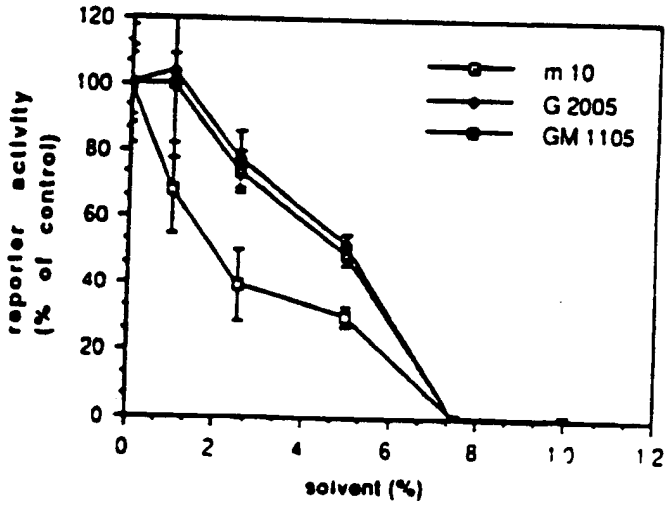

G 21
G 1002
G 2005
G 2071
G 2085
G 3014
G 3031
G/5637
5637
HL 60
M/HL 60
M 2071
M 2085
M 2086
GM 1073
GM 1081
GM 1088
GM 1090
GM 1098
GM 1105
GM/5637
5637

Figure 49

8 hours DMSO



8 hours ethanol



8 hours methanol

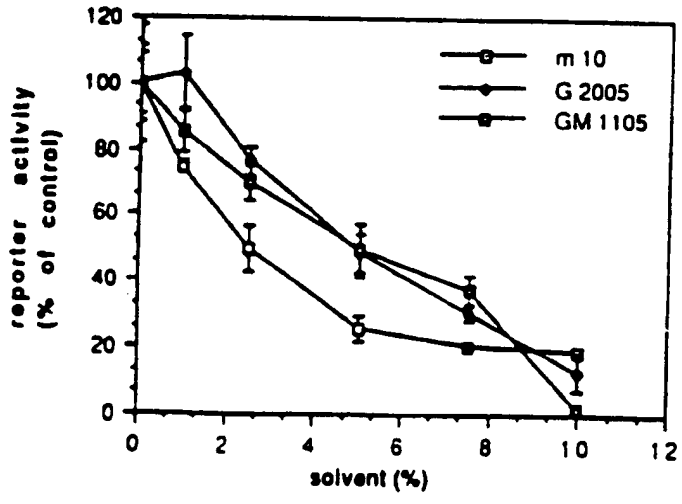


Figure 50

Signal Halflife

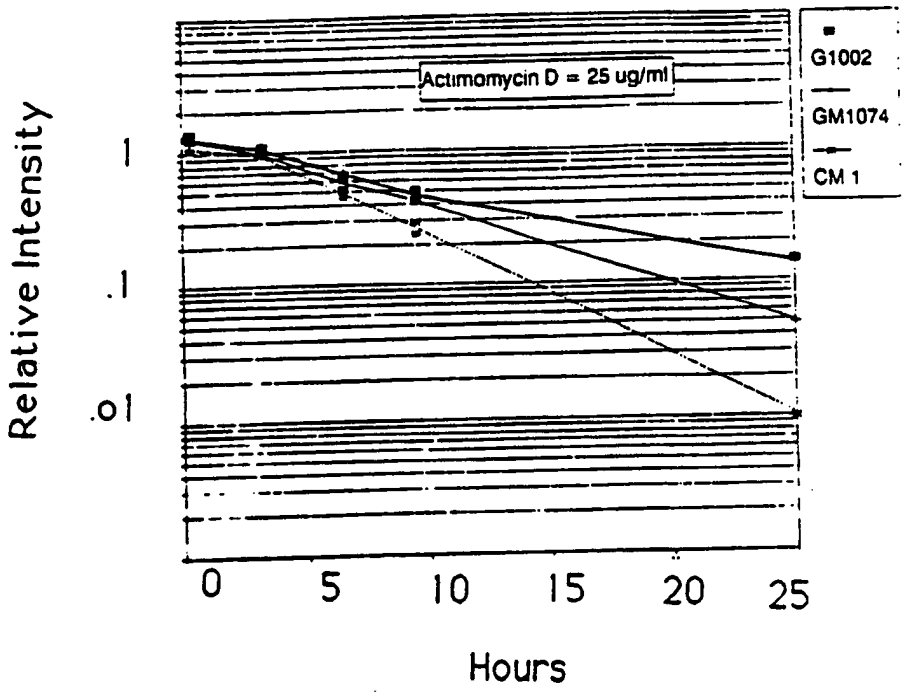


Figure 51

Ratios of Negative Controls

SP000011

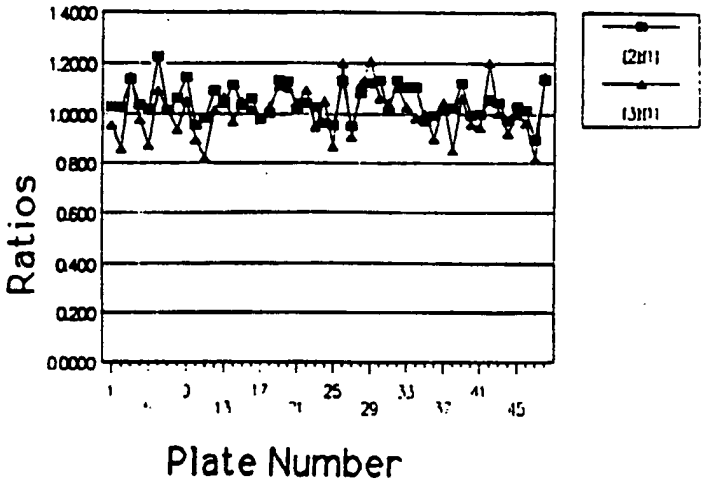


Figure 52

Average Negative RCOV

SP000011 - Avg.RCOV

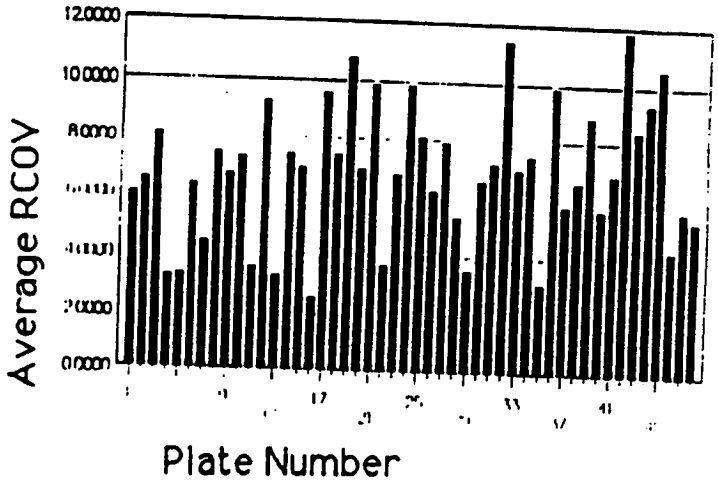


Figure 53

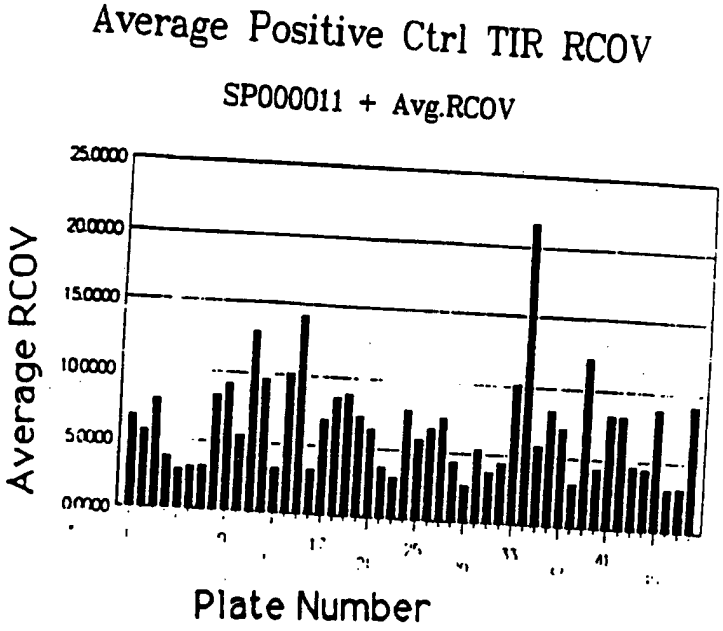

Average Positive Ctrl TIR RCOV
SP000011 + Avg.RCOV
25.0000
20.0000
15.0000
10.0000
5.0000
0.0000
Average RCOV
Plate Number

Figure 54

Positive Control TIR Signals

SP000011 Cell:MMT + Medians

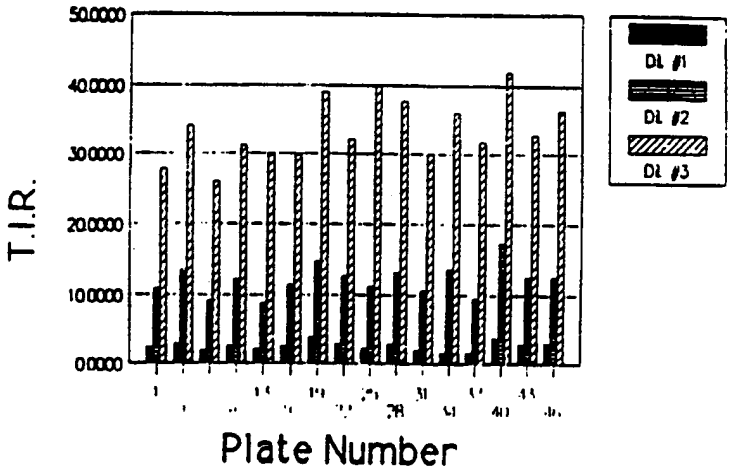


Figure 55

Positive Control TIR Signals

SP000010 Cell:MMT + Medians

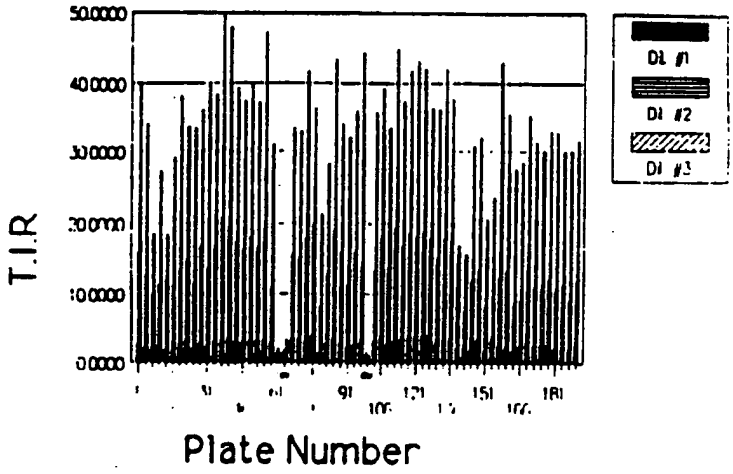


Figure 56

Examples of Primary Screen Lead Chemicals

Specific Transcriptional Inducers

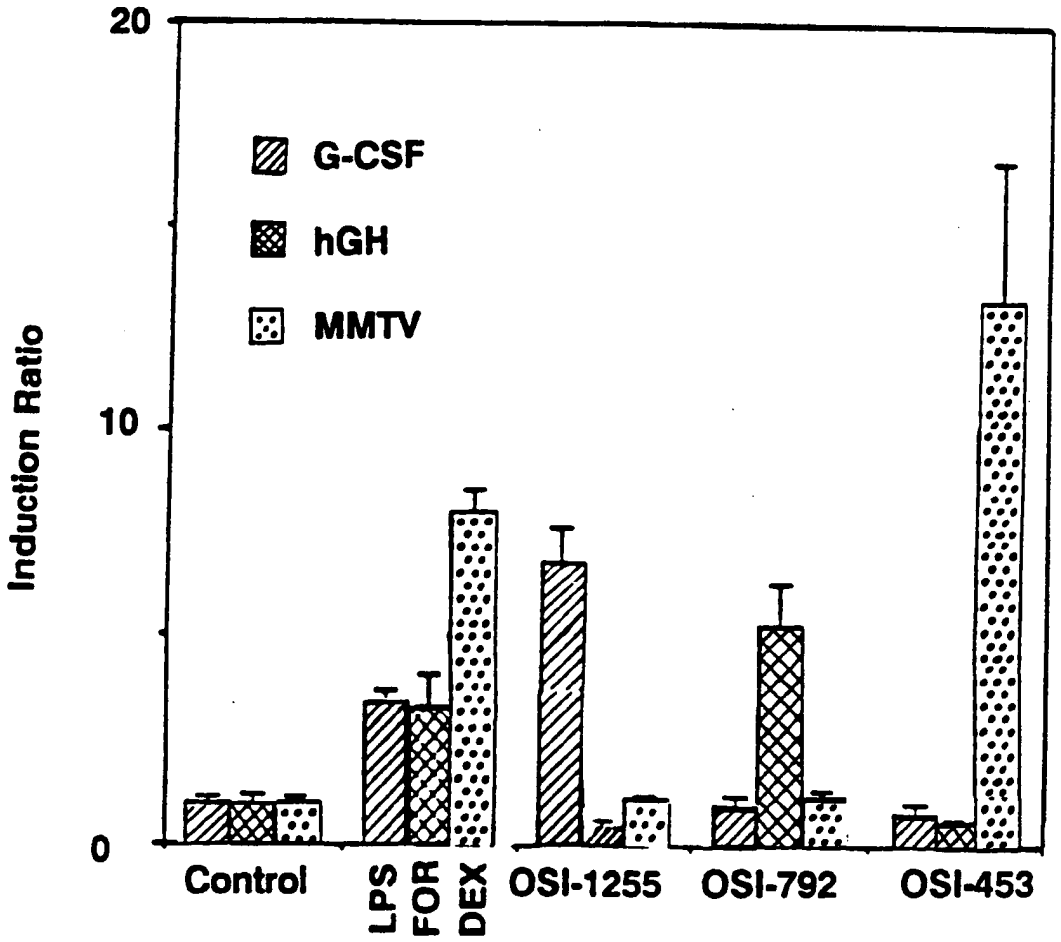


Figure 57

Examples of Primary Screen Lead Chemicals

Specific Transcriptional Inhibitors

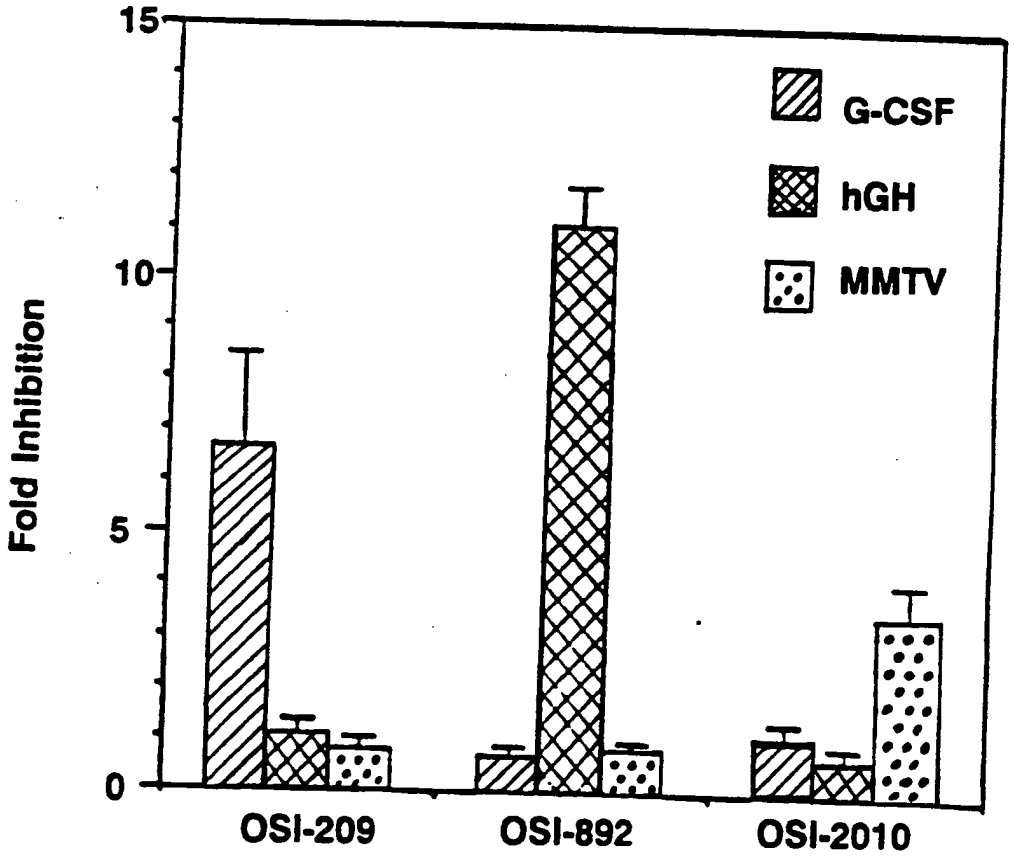


FIGURE 58

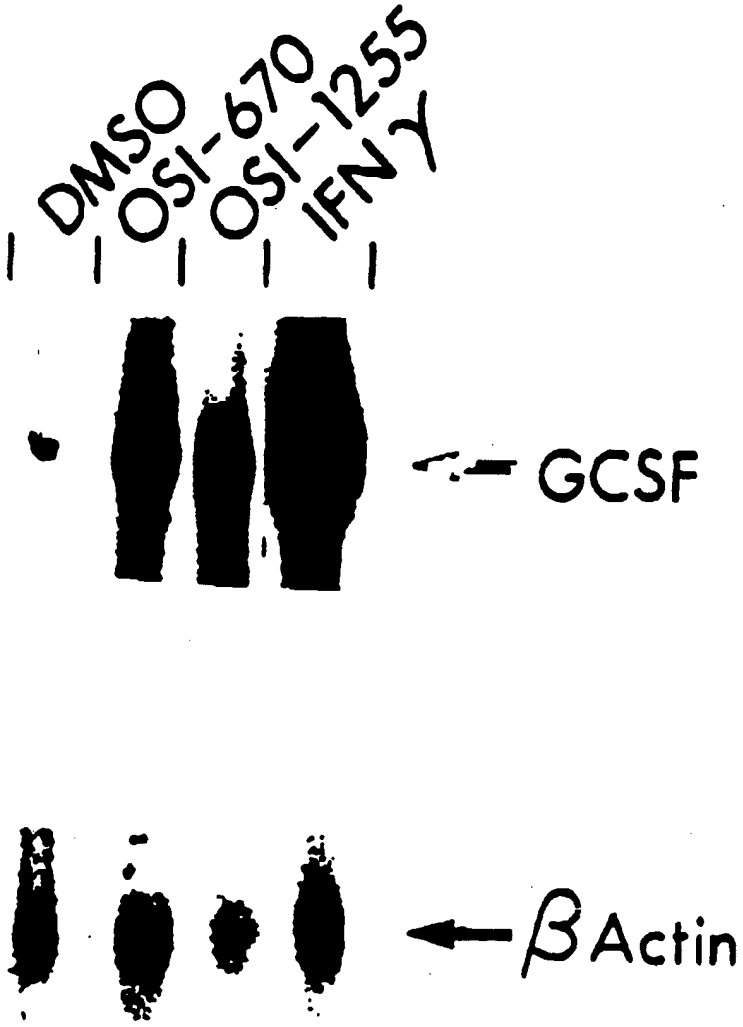


Figure 59

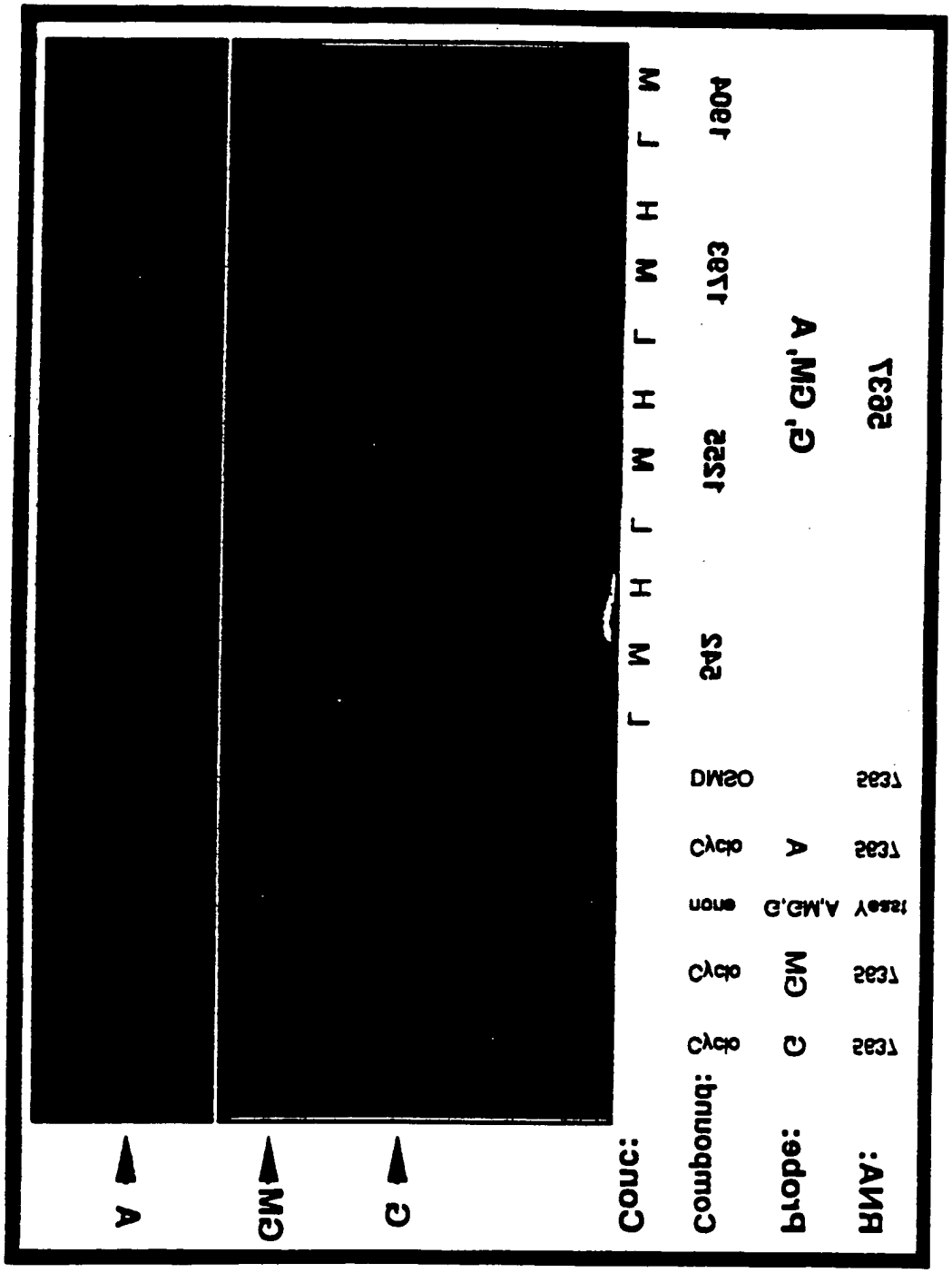


Figure 60

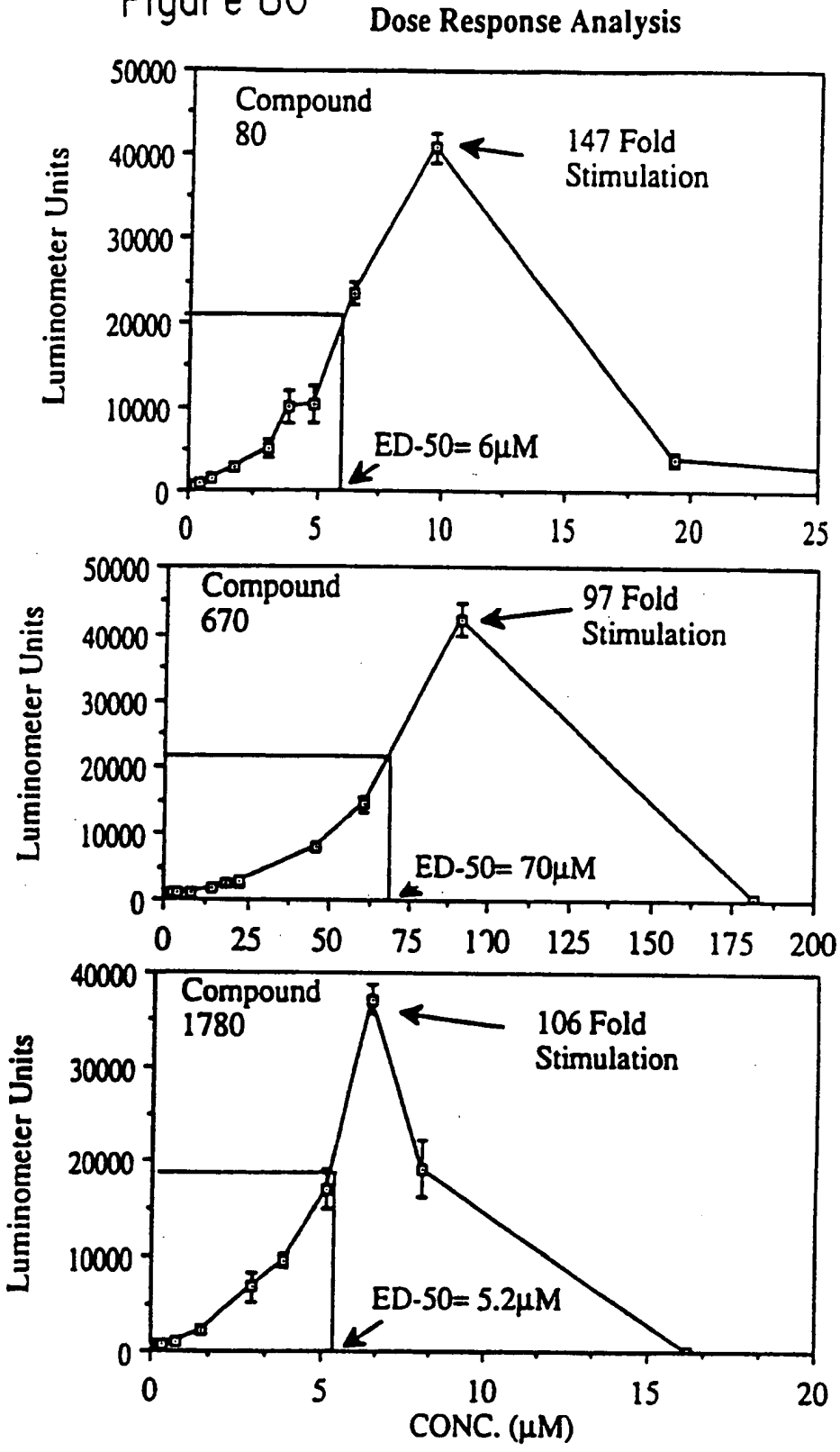

Dose Response Analysis
Compound 80
147 Fold Stimulation
ED-50= 6µM
Compound 670
97 Fold Stimulation
ED-50= 70µM
Compound 1780
106 Fold Stimulation
ED-50= 5.2µM
Luminometer Units
CONC. (µM)

Figure 61

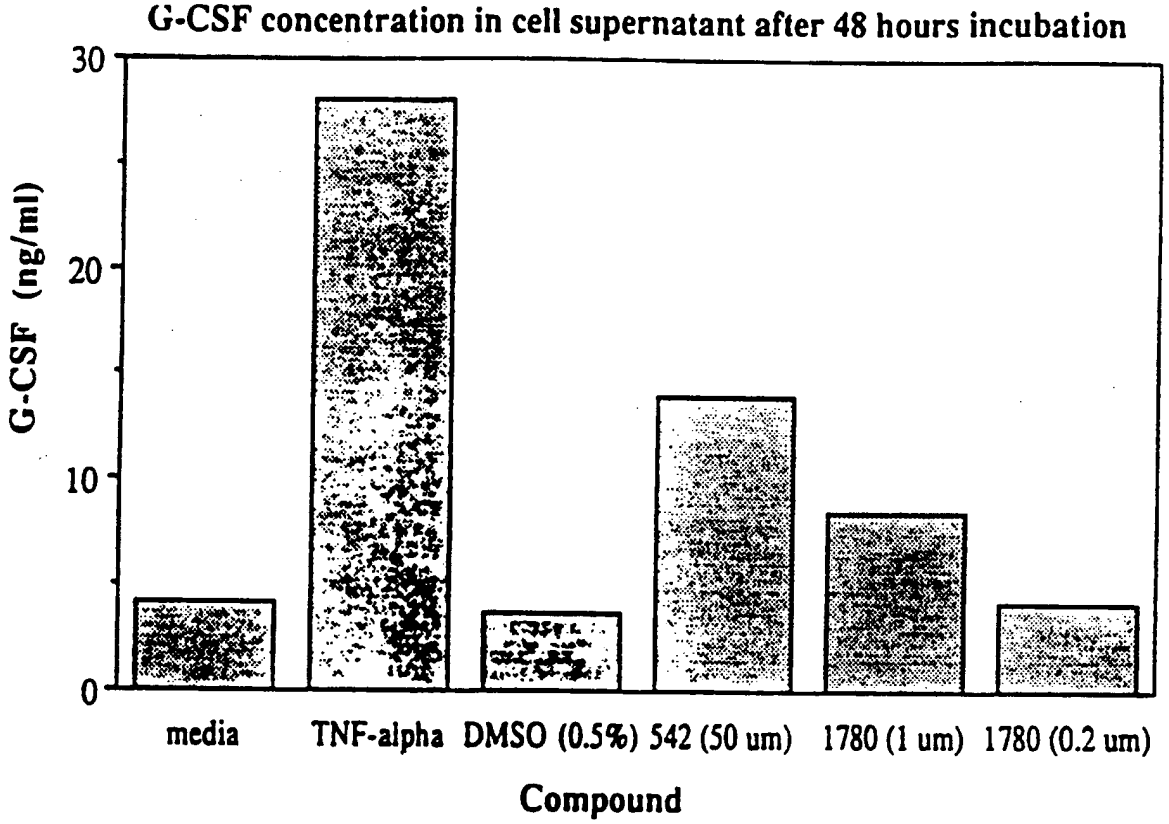

G-CSF concentration in cell supernatant after 48 hours incubation
G-CSF (ng/ml)
30
20
10
0
media
TNF-alpha
DMSO (0.5%)
542 (50 um)
1780 (1 um)
1780 (0.2 um)
Compound

Figure 62

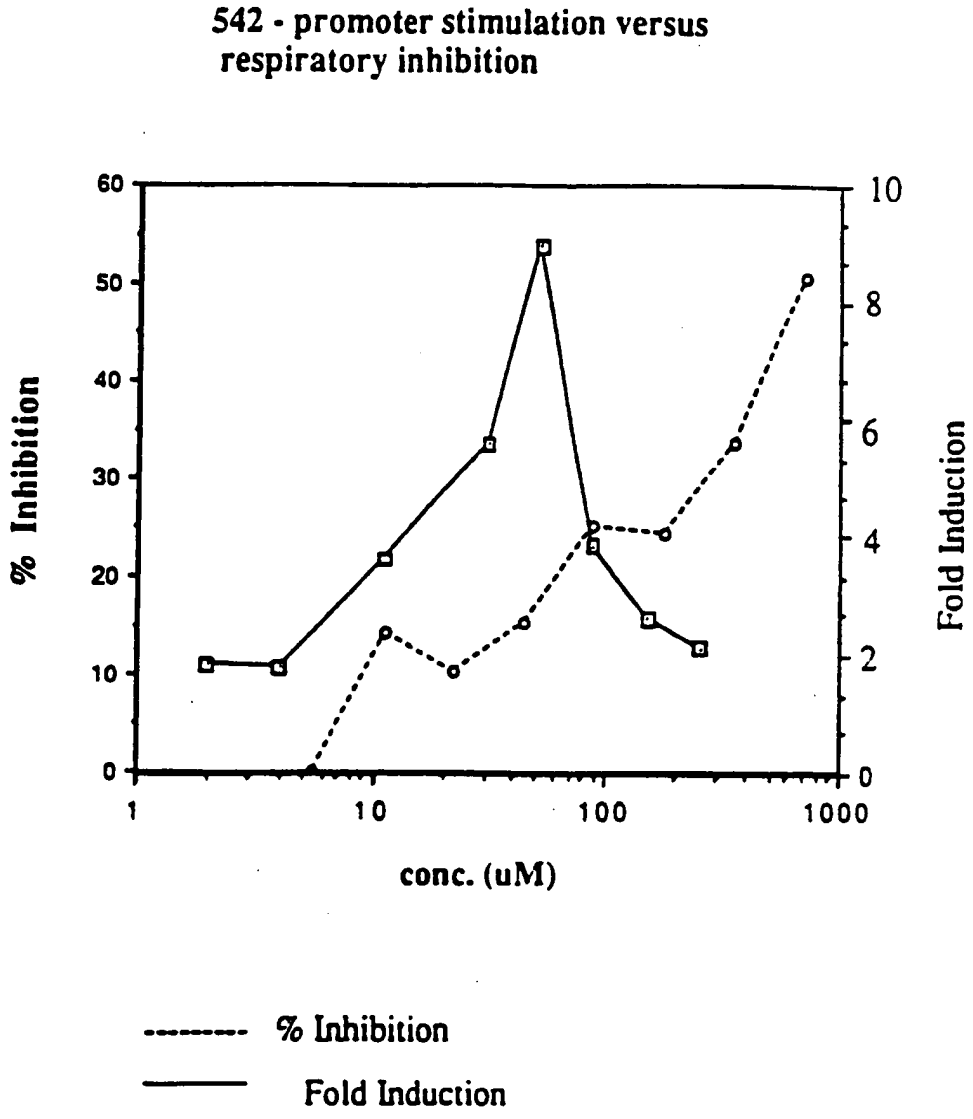
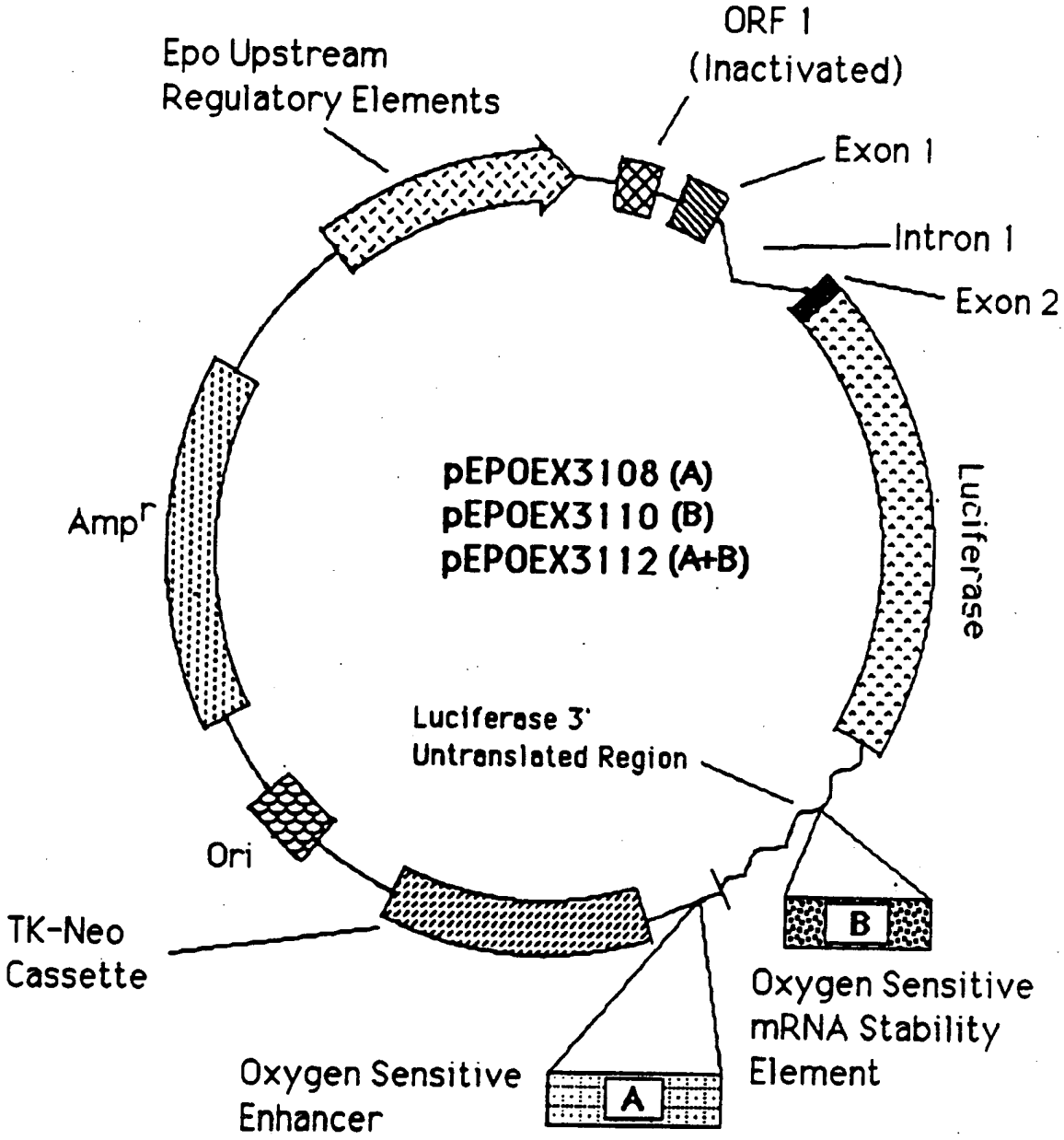

542 - promoter stimulation versus respiratory inhibition
% Inhibition
Fold Induction
conc. (uM)
60
50
40
30
20
10
0
10
8
6
4
2
0
1
10
100
1000
% Inhibition
Fold Induction

Figure 63. The Structure of pEPOEX3108 pEPOEX3110 and pEPOEX3112



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00451

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 7.1, 7.5, 8, 69.4, 69.5, 69.62, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col 1-10 and the examples.	1-23, 58-79
Y	US, A, 4,740,461 (Kaufman) 26 April 1988. See col 1-16 and the examples.	1-23, 58-79
Y	EP, A, 0,117,058 (Levinson et al.) 29 August 1984. See at least the abstract and pages 5-18.	1-23, 58-79
Y	Mol. Cell. Biol. vol. 7, no. 6, issued June 1987, Angel et al., "12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region", pages 2256-2266. See the abstract and the figures.	1-23, 58-79
Y	Proc. Natl. Acad. Sci. USA. Volume 83, issued May 1986, Kaushansky et al., "Genomic cloning, characterization, multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor". pages 3101-3105. See Figure 2, and page 3105.	1-23, 58-79

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
20 APRIL 1992	07 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Richard Fritsch for Christopher Low

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EMBO J. Volume 6, No. 9, issued September 1987, Ladner et al., "Human CSF-1: gene structure and alternative splicing of mRNA". pages 2693-2698. See at least pages 2693, 2696-2997.	1-23, 58-79
Y	EMBO J. Volume 6, No. 4, issued April 1987, Lefevre et al., "Tissue-specific expression of the human growth hormone gene is conferred in part by the binding of a specific trans-acting factor". pages 971-981. See at least page 971.	1-23, 58-79

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Proc. Natl. Acad. Sci. USA. Volume 82, issued November 1985, Lin et al., "Cloning and expression of the human erythropoietin gene". pages 7580-7584. See entire document.	1-23, 58-79
Y	EMBO J. Volume 5, No. 3, issued March 1986, Nagata et al., "The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor. pages 575-581. See at least the abstract.	1-23, 58-79
Y	Cell Volume 47, issued 10 October 1986, Yang et al., "Human IL-3 (multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. pages 3-10. See Figure 4, and page 6.	1-23, 58-79
Y	de Serres et al., "Chemial Mutagens. Principles and methods for their detection", published 1980 by Plenum Press (New York). pages 331, and 365-473. See at least pages 331, 367-369, 377.	1-23, 58-79
Y	Science Volume 227, issued 15 March 1985, Engebrecht et al., "Measuring gene expression with light". pages 1345-1347. See entire document.	1-23, 58-79
Y	Science Volume 236, issued 05 June 1987, Maniatis et al., "Regulation of inducible tissue-specific gene expression", pages 1237-1245. See pages 1237, 1239, 1240, 1243.	1-23, 58-79
Y	Molec. Cell. Biol. Volume 7, No. 2, issued February 1987, de Wet et al., "Firefly luciferase gene: Structure and expression in mammalian cells". pages 725-737. See the abstract, Fig. 1 and 3, pages 729-734.	1-23, 58-79
Y	Exp. Hematol., vol. 16, issued 1988, Bickel et al., "Granulocyte-Macrophage Colony-Stimulating Factor Regulation in Murine T·Cells and Its Relation to Cyclosporin A", pages 691-695. See entire document.	1-23, 58-79
Y	US, A, 4,601,978 (Karin) 22 July 1986, see entire document.	1-23, 58-79
Y	WO, A, 89/02472 (Shannon et al.) 23 March 1989. See entire document.	1-23, 58-79
Y	Cell, vol. 49, issued 19 June 1987, Angel et al., "Phorbol Ester-Inducible Genes Contain a Common Cis Element Recognized by a TPA-Modulated Trans-Acting Factor", pages·729-739. See entire document.	1-23, 58-79
Y	Science, vol. 230, issued 18 October 1985, Kawasaki et al., "Molecular Cloning of a Complementary DNA Encoding Human Macrophage-Specific Colony-Stimulating Factor (CSF-1)", pages 291-296, see entire document.	1-23, 58-79
Y	Proc. Natl. Acad. Sci. USA., vol. 81, issued August 1984, Kronke et al., "Cyclosporin A Inhibits T-Cell Growth Factor Gene Expression at the Level of mRNA Transcription", pages 5214-5218. See entire document.	1-23, 58-79
Y	Gene Amplification (Schimke, R. T., ed.), issued 1982, Mayo et al., "Altered Regulation of the Mouse Metallothionein I Gene Following Gene Amplification or Transfection", pages 67-73. See entire document.	1-23, 58-79

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y,P	US, A, 5,070,012 (Nolan et al.) 03 December 1991. See entire document.	24-51, 52-57
Y	US, A, 4,981,783 (Augenlicht) 01 January 1991 see entire document.	24-51, 52-57
Y	US, A, 4,806,463 (Goodchild et al.) 21 February 1989. See entire document.	24-41, 52-57

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12P 21/00, 21/02; C12N 5/00, 7/04, 15/00, 1/38; C12Q 1/66, 1/68, 1/00

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 7.5, 8, 69.4, 69.5, 69.62, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

II. FIELDS SEARCHED
Other Documents Searched:

USPTO APS - USPAT, JPOABS
DIALOG - BIOSIS, CHINESE PATENT ABSTRACTS, CLAIMS/USPATENTS, INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENT ABSTRACTS
Search Terms - mammal, toxicity, toxic, chemical?, carcinogen?, oncogen?, transcript?

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-23 and 58-79 drawn to a method of transcriptional modulation of a gene encoding a hematopoietic growth factor the expression of which is associated with a defined physiological or pathological effect where the cell or multicelular organism capable of expressing the gene is contacted with molecules which are not naturally present or expressed by the cells and which modulate expression of the gene product, are classified in Class 435, subclass 69.4 and various subclasses of Class 514.

II. Claims 24, 25, 27-51, and 53-57 drawn to a method of determining whether a molecule is capable of transcriptional modulation of expression of a gene encoding a hematopoietic growth factor by detecting the presence of a polypeptide coded for by a reporter gene are classified in Class 435, subclasses 7.1, 7.5, and 8.

III. Claims 26-41 and 52-57 drawn to a method of determining whether a molecule is capable of transcriptionally modulating expression of a gene coding for a hematopoietic growth factor by measuring the mRNA produced are classified in Class 435, subclass 6.